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(Regn. No. 28 OF 2007)

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Mahavadi, S., Rao, R.S.S.K. and Murthy, K.S. (2007). Cross-regulation of VAPC2 receptor internalization by m2 receptors via c-Src-mediated phosphorylation of GRK2. Regulatory Peptides, 139: 109-114.

Lehninger, A.L., Nelson, D.L. and Cox, M.M. (2004). Lehninger Principles of Biochemistry, (4th edition), W.H. Freeman & Co., New York, USA, pp. 73-111.

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In Silico Investigation on the Probable Macromolecular Drug Targets Involved in the Anti-Schizophrenia Activity of *Ocimum sanctum*

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Abstract

Despite the low prevalence of schizophrenia, it negatively impacts the quality of life for patients and their families. The present antipsychotics improve only the positive symptoms of the illness, but they do not affect the negative symptoms or cognitive impairment. Thus, there is no satisfactory and effective remedy available to prevent, manage, and cure schizophrenia. *Ocimum sanctum* is an Ayurvedic medicinal plant, the active constituents of the plant are known to have certain activities which might be useful in alleviating the symptoms of schizophrenia. Therefore, the objective of this study is to identify the active constituents of *Ocimum sanctum* with the highest affinity for the probable macromolecular drug targets involved in the aetiology of schizophrenia and, thereby determine the structural features of the ligands that involve in the interactions with the drug targets and propose some modifications for designing of new drugs. The phytochemicals present in *Ocimum sanctum* were filtered first based on their anti-schizophrenia activity and Lipinski's rule of 5. Then, the selected active constituents were subjected to molecular docking against the dopamine receptor, N-methyl-D-aspartate receptor, Gamma-aminobutyric acid receptor and phosphodiesterase 10A. Apigenin exhibited a particularly high binding affinity towards the four targets. Thus, apigenin can serve as a starting point for the designing of new compounds with the highest affinity for the target receptors.

Key words : Schizophrenia, Antipsychotics, *Ocimum sanctum*, Molecular Docking, Apigenin

1. Introduction

Schizophrenia is a mental disorder that affects the ability of an individual to think, feel, and behave. It is characterised by three symptomatic domains : positive symptoms, negative symptoms and cognitive dysfunction.The pathophysiology of schizophrenia is unclear but it has been postulated that the psychosis observed in schizophrenia is attributed to the hyperactivation of mesolimbic pathway and dysfunction of the mesocortical pathway leading to an imbalance in the dopaminergic, serotonergic, GABAergic as well as glutamatergic neurotransmission in certain regions of the brain. In addition, other factors can also contribute to the development of psychosis, i.e. heredity, oxidative stress, N-methyl-D-aspartate (NMDA) receptor antagonists, drug abuse and traumatic injury. The positive symptoms are considered the most common symptoms of schizophrenia. These symptoms are effectively treated with the currently available antipsychotics. Whereas the negative symptoms and cognition dysfunction do not respond to the available medications. Thus, there is no satisfactory and effective remedy available to prevent, manage and cure schizophrenia (1, 2).

Recently, the major attention has been directed toward exploring and studying the therapeutic potential of natural herbs in the management of schizophrenia. *Ocimum sanctum* (also known as tulsi or holy basil) is one of the plants that is renowned for its therapeutic uses. It is an Ayurvedic medicinal plant which is believed to act as potent adaptogenic herb that protect the body from the effects of various stressors (3). Despite the numerous therapeutic values of *Ocimum sanctum,* the attention was drawn mainly to certain activities which might be useful in alleviating the symptoms of schizophrenia. These activities include antioxidant and anti-stress activities, memory and cognition-enhancing activities and neuroprotection activities. Therefore, recent research has been driven to investigate the therapeutic potential of *Ocimum sanctum* in treating schizophrenia (4-6).

Computational (*in silico*) methods have been applied widely and frequently in the process of drug discovery and development to help in the selection of potent lead molecule, thereby minimise the failures during the late stage of clinical development (7). The aim of the present study was to identify the potential anti-schizophrenia phytochemical compounds present in the *Ocimum*

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sanctum. Molecular docking has been used to study the protein-ligand interactions by predicting the preferred orientation and free energies of binding between the active constituents of *Ocimum sanctum* (ligands) and the target receptors (macromolecules) which are known to be related to the pathophysiology of schizophrenia.

2. Materials and Methods

Selection of phytochemicals from Ocimum sanctum

A Literature review has been conducted to identify a number of phytochemicals present in various parts of *Ocimum sanctum.* All these phytochemicals were selected from reported scientific literature based on their pharmacological activities. Only the active constituents were further filtered according to the Lipinski's rule of 5 and polar surface area (PSA). The inclusion criteria are presented in **Table 1** (8, 9). The violation of either one of these properties will result in the exclusion of the active constituent.

Table 1: Lipinski's rule of 5 and polar surface area

S.No.	Inclusion Criteria
1	Molecular weight (MW) ≤ 500 Dalton (g/mol)
2	Hydrophobicity (logP) ≤ 5
3	Number of hydrogen bond donor (nOHNH) ≤ 5
4	Number of hydrogen bond acceptor (nON) ≤ 10
5	Number of rotatable bonds (nrotb) < 8
6	Polar surface area (PSA) of 20 – 90 $Å^2$

Preparation of proteins

The three dimensional (3D) structures of dopamine (D2) receptor, N-methyl-D-aspartate (NMDA) receptor, gamma-aminobutyric acid GABAA receptor, and phosphodiesterase (PDE) 10A were downloaded from the Protein Data Bank (PDB) and used as macromolecular targets for the docking study **(Table 2)** (10).

Table 2 : Macromolecular targets with their respective PDB ID, co-crystallised ligands, and standard compounds.

Macromolecular Targets	PDB ID	Co-crystallized Ligands	Standard Compounds
Dopamine (D_2)	6CM4	Risperidone	Risperidone
N-Methyl-D-Aspartate (NMDA)	5U8C	PEAQX	PEAQX
Gamma-Aminobutyric Acid ($GABA_A$)	6D6T	Flumazenil	Alprazolam
Phosphodiesterase(PDE) 10A	2WEY	Papaverine	Mardepodect

The protein structures were prepared for docking by removing the co-crystallised ligands first using Biovia Discovery Studio (DS) (11). Those co-crystallised ligands were saved as separate files to be used as standards for the docking studies. The protein structures were imported into AutoDock where AutoDock Tools (ADT) 1.5.6 was used to prepare each protein by removing the water molecules, adding the hydrogen atoms, and Gasteiger charges (12).

Preparation of ligands

The two dimensional (2D) structures of the ligands (the standard compounds and the selected active constituents) were drawn using ACD/ChemSketch software (13). Followed by conversion the ligands into their 3D structures using Open Babel (14). The 3D structures of the ligands were imported into AutoDock where they were subjected to the addition of charges. Then the rotatable bonds were set by ADT and all the torsions were allowed to rotate for the ligands (12).

Identification of the proteins' binding sites

The amino acid residues of each prepared protein which interact with its co-crystallised ligand were determined by using Biovia DS (11). The binding site of the protein was identified based on the amino acid residues involved in the interactions. ADT was used to determine the Grid Box that covers the entire identified binding site of the protein (12).

Molecular docking

AutoDockVina 1.1.2 was used to perform molecular docking of the prepared ligands against the binding sites of the prepared macromolecular drug targets (12). During the docking process **(Figure 1)**, flexible ligands were docked into a rigid binding site of the protein structure. The ligands were ranked based on their affinity (binding energy) to the target receptor in which ligands with lower binding energies were ranked higher. Additionally, the orientation of the top ligands and the mode of interactions with each drug target were studied using Biovia DS (11).

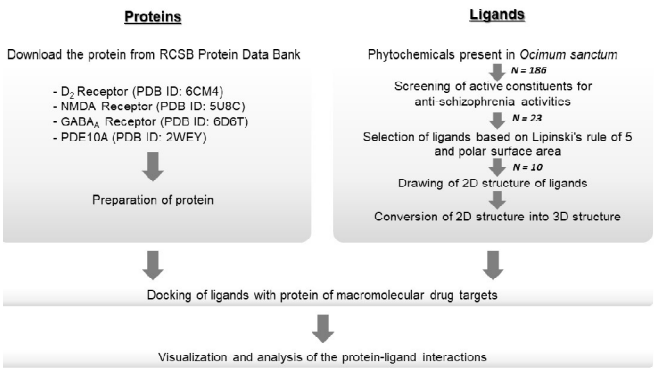

Figure 1 Flow chart of the molecular docking study.

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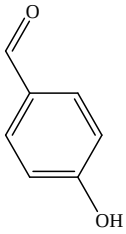
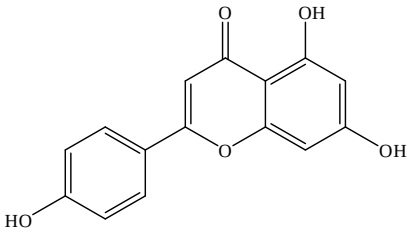
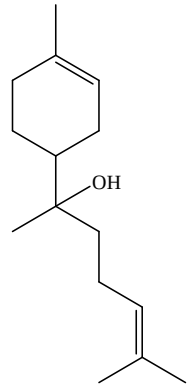
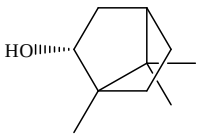
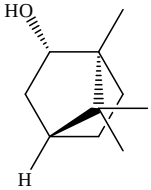
3. Results and Discussion

Selection of phytochemicals from Ocimum Sanctum

The phytochemicals were selected based on inclusion criteria mentioned previously. The selected compounds along with their lipinski's rule of 5 and PSA values are presented in **Table 3.**

Table 3 : The active constituents of *Ocimum sanctum* with their lipinski's rule of 5 and PSA values.

No.	Ligands	MW	Log P	nOHNH	nON	nrotb	PSA
1	4-Hydroxybenzaldehyde 	122.12	1.25	1	2	1	37.30
2	Apigenin 	270.24	2.46	3	5	1	90.89
3	α-Bisabolol 	222.37	4.68	1	1	4	20.23
4	Borneol 	154.25	2.35	1	1	0	20.23
5	Isoborneol 	154.25	2.35	1	1	0	20.23

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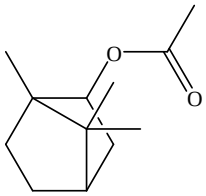
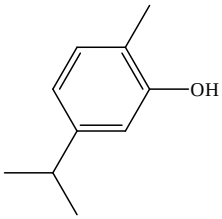
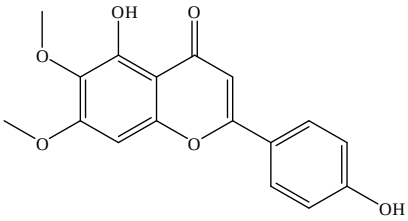
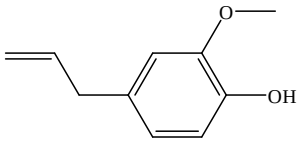
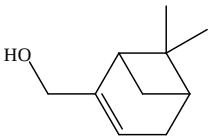
6	Bornyl acetate 	196.29	3.05	0	2	2	26.3
7	Carvacrol 	150.22	3.81	1	1	1	20.23
8	Cirsimaritin 	314.29	2.79	2	6	3	89.14
9	Eugenol 	164.20	2.10	1	2	3	29.46
10	Myrtenol 	152.24	2.30	1	1	1	20.23

Molecular Docking

186 phytochemicals are presented in *Ocimum sanctum* (Supplementary Materials). These phytochemicals were screened based on their pharmacological properties and Lipinski's rule of 5. As a consequence, only ten active constituents have been selected to be docked against the probable macromolecular drug targets of schizophrenia, D2 receptor, NMDA receptor, GABAA receptor, and PDE10A receptor. The docking results are presented in **Table 4.**

As can be seen in Table 4, all the active constituents displayed low to intermediate affinities toward the four receptors except apigenin which exhibited the highest affinity (binding energy $\leq$ -9.5 Kcal/mol) to the target receptors followed by cirsimaritin (binding energy $\leq$ -8.2 Kcal/mol) then α-bisabolol (binding energy $\leq$ -7.0 Kcal/mol). However, the binding energies that displayed by the four standards were the lowest in comparison to the active constituents of *Ocimum sanctum*, thus these standards had the highest affinities toward the target receptors.

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Table 4 : Binding energy of the standard compounds and the active constituents of *Ocimum sanctum* with the probable macromolecular drug targets of schizophrenia.

No.	Ligands	Binding Energy (kcal/mol)			
		D_2Receptor	NMDA	$GABA_A$	PDE10A
Standard compound for each receptor					
1	Risperidone	-12.0	-	-	-
2	PEAQX	-	-10.6	-	-
3	Alprazolam	-	-	-11.2	-
4	Mardepodect	-	-	-	-9.8
Active constituents of *Ocimum sanctum*					
5	4-Hydroxybenzaldehyde	-5.7	-5.2	-6.2	-5.6
6	Apigenin	-10.3	-9.5	-9.7	-9.6
7	α-Bisabolol	-8.6	-7.3	-7.8	-7.0
8	Borneol	-6.3	-5.9	-6.4	-5.2
9	Isoborneol	-6.6	-6.0	-6.9	-5.4
10	Bornyl acetate	-7.2	-6.5	-6.9	-6.0
11	Carvacrol	-7.0	-6.2	-7.0	-6.8
12	Cirsimaritin	-9.5	-8.2	-9.3	-8.9
13	Eugenol	-6.5	-6.0	-6.9	-6.3
14	Myrtenol	-6.4	-6.1	-5.9	-5.3

Apigenin

Apigenin is a plant-derived flavonoid that belongs to the flavone class. It can be found abundantly in herbs, fruits and vegetables. The structure of apigenin contains a fused benzene ring (Ring A) and oxygen-containing heterocycle (Ring C) that form a benzopyrone moiety, besides the phenyl ring (Ring B) at position 2. The benzopyrone moiety and the phenyl ring constitute the phenylchromane nucleus of apigenin (15). It is substituted with three hydroxyl groups at positions 4', 5, and 7 forming a trihydroxyflavone with the molecular formula of $C_{15}H_{10}O_5$ **(Figure 2)**.

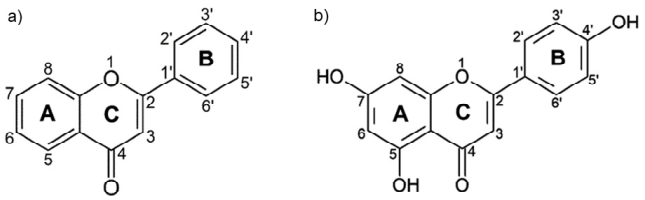

Figure 2 : a) General structure of flavones. b) Structure of apigenin (4',5,7-trihydroxy-flavone)

Based on the docking results, apigenin displayed high affinity to all macromolecular drug targets involved in schizophrenia. Therefore, the mode of interactions of apigenin with the target receptors has been studied thoroughly to identify the amino acids that are involved in the interactions and the bonded and non-bonded interactions that contribute to the strength of binding. Furthermore, the mode of interactions of the standard compounds has been studied as well to illustrate the important amino acids that are essential for the binding affinity and selectivity.

Dopamine (D2) receptor

Risperidone is a benzisoxazole derivative that belongs to the atypical antipsychotic medications. It is widely prescribed as a treatment for the positive symptoms of schizophrenia due to its potent D2 receptor antagonism activity (16, 17). Thus, risperidone has been used as a standard compound for assessing the docking results of the active constituents of *Ocimum sanctum* with D2 receptor. Based on the docking results, risperidone has shown the highest binding affinity (binding energy -12.3 kcal/mol) to the D2 receptor. The high affinity might be due to the strong interactions between the ligand and the active site's residues of D2 receptors. These interactions involve two hydrogen bonds between the oxygen atom in benzisoxazole and the residues Thr119 and Ser197 and n-n T-shaped interactions between the pyrimidone ring and phenyl ring of the ligand and the aromatic rings of Trp100 and Trp386, respectively. Additionally, the fluorine group of risperidone displays a unique interaction

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(halogen interaction) with the residue Cys118 of the receptor. This carbon-fluorine interaction has similar structural significance as the week hydrogen bond. The benzisoxazole moiety of risperidone is important for tethering the ligand in the active site of the D2 receptor. This moiety extends into a deep binding pocket, that form a sub-pocket underneath the orthosteric site of the D2 receptor, thereby contributing to the significant binding affinity and the strength of binding **(Figure 3)** (18).

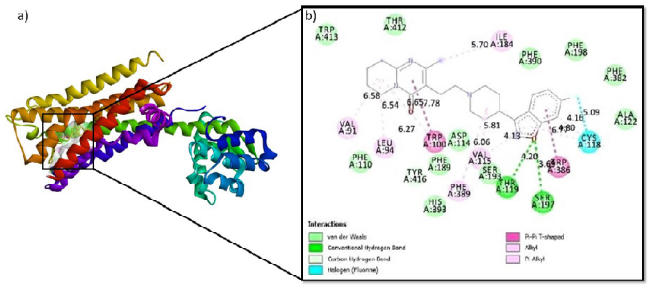

Figure 3. Molecular docking of risperidone with D2 receptor. a) ligand-binding site of D2 receptor. b) 2D representation of D2-risperidone complex interactions.

Apigenin showed the highest binding affinity (-10.3 kcal/mol) to D2 receptor in comparison to the rest of the active constituents that have subjected to molecular docking. The ligand has involved in three hydrogen bonds with the active site residues that contribute to the strong affinity to the D2 receptor. These hydrogen bonds form between the two hydroxyl groups and the carbonyl group of apigenin and the residues Cys118, Tyr416 and Ser193, respectively. The residues Phe198, Trp386, and Phe390 stacked on the phenyl ring of apigenin vertically to form three n-n T-shaped interactions indicating the significance of the phenyl ring (ring B) in the deep pocket-binding of D2 receptor **(Figure 4)**.

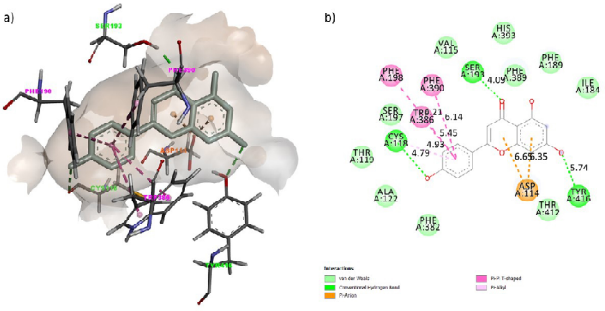

Figure 4. Binding interactions of D2-apigenin complex. a) 3D representation. b) 2D representation.

Previous studies have illustrated the important role of Asp114, Ser193, and Ser197 residues in the binding of D2 receptor agonists and antagonists (17, 19, 20). Therefore, tertiary nitrogen (eg. ethyl piperidine) has been suggested to be added as a bridge between position 1' and position 2 to lengthen the molecule hence strengthen the binding. In this case, a salt bridge will be formed between the basic nitrogen atom (positively charged) and the acidic Asp114 residue (negatively charged). Besides, the basic nitrogen will contribute to the reduction of the logP value, thus enhancing brain penetration. This structural modification will add some advantage to apigenin (9).

N-Methyl-D-Aspartate (NMDA) receptor

The NMDA receptor hypofunction is strongly implicated in the aetiology of schizophrenia. Thus, NMDA receptor agonist is required to reverse this effect. PEAQX is a competitive NMDA receptor antagonist. This compound has been used in the docking study for the comparison of the docking results due to unavailability of NMDA receptor agonist (1, 21, 22). The binding mode of PEAQX within the active site of the NMDA receptor showed that the ligand interacts with the receptor via seven hydrogen bonds. These hydrogen bonds form between the nitrogen atom and two carbonyl groups of quinoxaline-dione moiety and the residues Ser114, Thr116, Arg121 and Ser173 from one side and the three oxygen atoms of the phosphoryl group and the residues Ser173, Thr174 and Tyr214 from the other side. All the amino acid residues act as hydrogen bond donors. Moreover, the phenyl ring of quinoxaline-dione moiety interacts with the aromatic ring of His88 and the bromophenyl interacts with the Thr116 **(Figure 5)**.

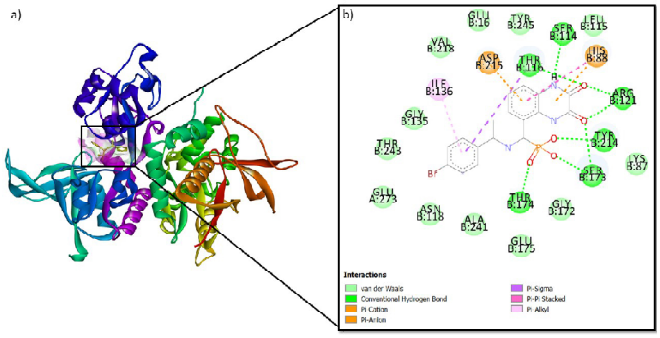

Figure 5. Molecular docking of PEAQX with NMDA receptor. a) ligand-binding site of NMDA receptor. b) 2D representation of NMDA-PEAQX complex interactions.

On the other hand, apigenin interacts with the NMDA receptor through six hydrogen bonds. The hydrogen bonds form between the carbonyl group and the three hydroxyl groups of the ligand and the residues Ser173, Thr174, Tyr214, Tyr245, Glu16 and Ala241, respectively **(Figure 6)**. Besides, the phenol ring of apigenin interacts with the residue Thr116, similar interaction was observed with PEAQX.

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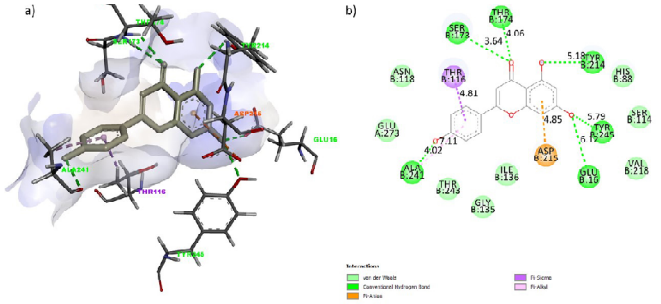

Figure 6. Binding interactions of apigenin with NMDA receptor. a) 3D representation. b) 2D representation.

Both PEAQX and apigenin exhibited similar affinity to NMDA receptor (binding energy -10.60 Kcal/mol for PEAQX and -9.5 Kcal/mol for apigenin). The high binding affinity is attributed to the strong interactions with the binding site residues in which both ligands interact with the residues Ser173, Thr174, and Tyr214 through hydrogen bonds. These hydrogen bonds are important for stabilising the ligands in the active site of the NMDA receptor.

Two studies have studied the binding mode of NMDA receptor antagonists including PEAQX as glutamate site ligand. They found that the bromophenly group interacts with the carboxylate group of Glu781 from GluN1 (different residue number is due to the differences between human and rat NMDA receptor). This interaction displaces three water molecules, results in water-mediated polar interaction at Glu781 of GluN1 and Ser689 and Thr690 of GluN2A (23, 24). Based on this finding, the replacement of the hydroxyl group attached to the phenyl ring (ring B) of apigenin with bromine group could increase the water-mediated polar interaction upon the displacement of water molecules by interaction of bromophenyl.

Gamma-aminobutyric acid GABAA receptor

Alprazolam is a short-acting benzodiazepine that binds to specific sites on the GABAA receptor. The binding of alprazolam to these sites (known as benzodiazepine binding sites) modulates the effect of GABAA receptors and, thus, of GABAergic neurons resulting in sedative, hypnotic and anxiolytic properties. In this study, alprazolam has been used as a standard compound where it displayed the highest binding affinity towards the GABAA receptor (binding energy -11.2 Kcal/mol). The ligand involved in several interactions with the benzodiazepine binding site of the GABAA receptor in which the nitrogen atom of the triazole ring forms a hydrogen bond with residues Thr142. The ligand acts as hydrogen bond acceptor where it accepted the hydrogen atom from Thr. Additionally, the phenyl ring of the ligand interacts with the aromatic ring of Phe77 and Tyr58 and the triazole ring interacts with the Lys156. Furthermore, the chloride group displays an interaction (halogen interaction) with the residue Gln204 **(Figure 7)**. All these interactions contribute to the strength of binding and the high affinity of alprazolam towards the benzodiazepine binding sites.

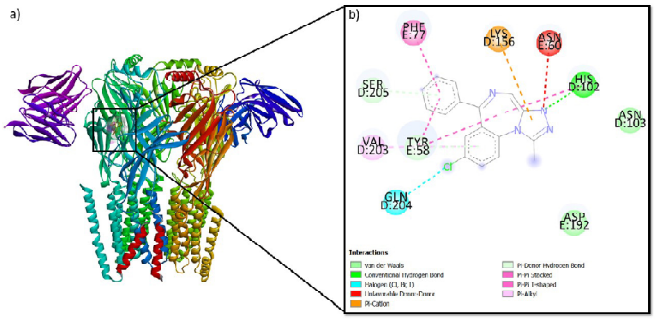

Figure 7. Molecular docking of alprazolam with GABAA receptor. a) ligand-binding site of GABAA receptor. b) 2D representation of GABAA-alprazolam complex interactions.

The binding mode of apigenin within the benzodiazepine binding site of GABAA receptor showed that the two hydroxyl groups and the carbonyl group of the ligand involve in four hydrogen bonds with the residues His102, Ser159, and Ala161, respectively. Four aromatic interactions can be observed between the chromene moiety of the ligand and the aromatic rings of the residues Tyr160 and Tyr210 **(Figure 8)**. Besides, the dihydropyranone ring of the chromene moiety involves in an interaction with the sulfur atom of Met130 (Pi-Sulfur interaction).

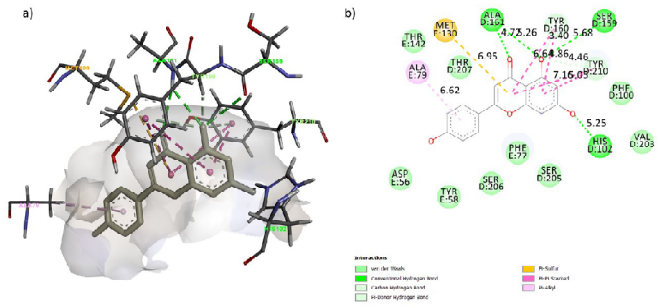

Figure 8. Binding interactions of apigenin with GABAA receptor. a) 3D representation. b) 2D representation.

In order to increase the binding affinity of apigenin to the benzodiazepine binding site, an amino group can be added at position 3. This group will interact via a hydrogen bond thus increasing the ligand's affinity and strengthening the binding.

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Phosphodiesterase (PDE) 10A

Mardepodect, also known as MP-10, is a selective inhibitor of PDE10A that has been developed by Pfizer for the treatment of schizophrenia. This compound showed the lowest binding energy to PDE10A (-9.8 kcal/mol). The low binding energy is attributed to the strong interactions between the protein, PDE10A, and the ligand. Mardepodect forms a hydrogen bond with Tyr524 where the Tyr donated the hydrogen atom to the ligand. Two aromatic interactions have been observed between the quinoline group of the ligand and the aromatic rings of Phe696 and Phe729. Moreover, the quinoline group occupies the hydrophobic clamp (P-clamp) of PDE10A in which the Phe696 and Phe729 are on one side and Ile692 on the other side **(Figure 9)**. Occupation the P-clamp is very important for stabilizing the ligand in the active site.

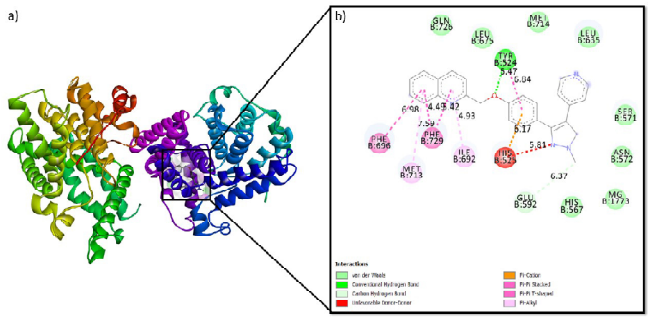

Figure 9. Molecular docking of mardepotect with PDE10A. a) ligand-binding site of PDE10A. b) 2D representation of PDE10A-mardepotect complex interactions.

In contrast to mardepodect, apigenin interacts with the active site residues of PDE10A through four hydrogen bonds. These hydrogen bonds form between the carbonyl group and the three hydroxyl groups of the ligand and the residues Tyr542, Gln726, Asp674 and Leu635, respectively. Apigenin also occupies the P-clamp and interacts with the residues Phe696, Phe729 and Ile692. The phenol ring of the ligand shows an interaction with the aromatic ring of Phe729. Furthermore, apigenin shows unique interactions with the metal ions Zn^{+2} and Mg^{+2} at the active site of PDE10A **(Figure 10)**. These interactions, hydrogen bonding with the invariant glutamine, Gln726, hydrophobic interactions with the three residues that form the P-clamp and the interactions with the metal ions contribute to the high affinity of the ligand to PDE10A.

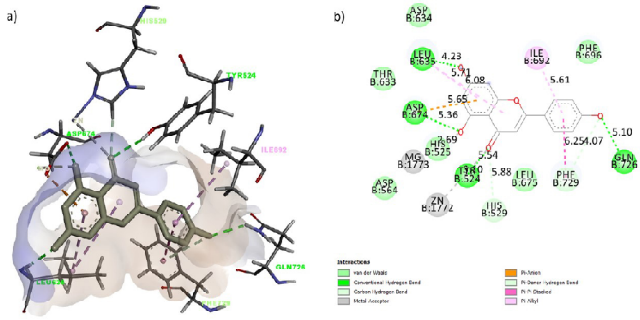

Figure 10. Binding interactions of apigenin with PDE10A. a) 3D representation. b) 2D representation.

An *in silico* study conducted by Wu Q. et al. to investigate the various quinoline derivatives as potent and selective PDE10A inhibitors using 3D-QSAR, molecular docking and molecular dynamics simulations methods has been reported. The authors have found that the bulky substituents at position 8 and around the pyridine ring (ring D) increase the biological activity of the inhibitor **(Figure 11)**. The replacement of the hydroxyl group at position 8 with a bulky group declines the inhibitory activity. The introduction of a heterocyclic ring containing a hydrogen bond acceptor atom at position 18 on the ring D is desirable to enhance the binding affinity and the inhibitory potency. And finally, the hydrophobic interactions with Ile692 and Met713, as well as the aromatic interactions with Phe696 and Phe729 are essential factors to increase the binding affinity (25).

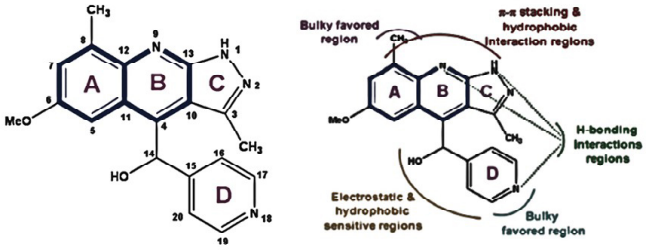

Figure 11. The structural features of quinoline derivatives indicated by in Wu Q et al.

Apigenin fulfilled the key interactions including hydrogen bonding with invariant Gln726, and hydrophobic contacts with Ile692, Phe696 and Phe729 and the aromatic interaction with Phe729. Modification can be performed to increase the binding affinity of apigenin by replacing the hydroxyl group at position 7 on ring A with a heterobenzene ring containing a hydrogen bond acceptor atom.

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4. Conclusion

The docking results of the ten active constituents of *Ocimum sanctum* with the four macromolecular drug targets involved in the pathophysiology of schizophrenia have shown that apigenin possess the highest affinities towards the four targets. The high affinities are attributed to the hydrogen bonding interactions, aromatic interactions and the hydrophobic interactions between the ligand and the receptors. All these interactions are important for stabilising the ligand in the active site of the target receptor. This study has highlighted the binding mode and the binding interactions between apigenin and the four receptors, thus, may serve as a starting point for the identification of a potential treatment for schizophrenia from natural sources. Further structure modifications can be performed to improve the key interactions with each receptor to enhance the binding affinity and design a new compound that can act as an anti-schizophrenic drug.

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Comparative Study on Antioxidant and Anti-Inflammatory Activities of Red and Brown Species of *Areca catechu* L. Nut Extracts

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Abstract

The bioactive products from medicinal plants have rich sources of secondary metabolites helpful to the treatment of several biological applications. Traditionally, areca nuts are the medicinal source that grows in India, Bangladesh, Sri Lanka, Myanmar, Taiwan, the tropical Pacific region, and the parts of East Africa. Several investigations identified the secondary metabolites of *Areca catechu* species inhibits anti-cancer activity, anti-inflammatory activity, anti-diabetic activity, and anti-microbial activity, respectively. The present study was aimed to investigating the phytoconstituents along with in vitro antioxidant activity and potential anti-inflammatory activity of red and brown varieties of ethanol extracts of areca nut for the inhibition of nitric oxide production in LPS-stimulated RAW 264.7 cells. The qualitative phytochemical analysis and thin layer chromatography differentiate the bioactive components. The ethanol extract of both the species areca nut red and brown varieties have identified the phytoconstituents such as tannins, cardiac glycosides, flavonoids, steroids, terpenoids, proteins, and phenolic compounds, respectively. The TLC fingerprinting observed six bands in the red areca nut and five bands were identified in brown areca nut. The antioxidant activity of areca nut extracts was performed by DPPH and ABTS radical scavenging assay. The IC50 values of DPPH assay from brown and red areca nut was found to be 24.54 ± 0.14 µg/ml, and 28.26 ± 0.11 µg/ml followed by the standard ascorbic acid shows 12.14 ± 0.17 µg/ml, respectively. The effective brown areca nut and the moderate effects of red nut were found to be 26.55 ± 0.08 µg/ml, 32.24 ± 0.01 µg/ml, and the standard ascorbic acid showed 6.10 ± 0.01 µg/ml, respectively. Significantly, the MTT assay shows the maximum concentrations of viable cells from both the extracts on RAW 264.7 cells. The LPS-induced nitric oxide production assay in RAW 264.7 cell line revealed that the brown areca nut extract reduces the nitric oxide level than red areca nut. The maximum inhibitory activity of brown areca nut extract from GC-MS analysis shows the following active constituents i.e. (2S,3S)-(-)-3-Propyloxiranemethanol, Propanedioic acid, propyl, 1,1-Dodecanediol, diacetate, 2-Nonenoic acid, Carbromal, 3-Nonenoic acid, Pentanoic acid, 2-(Aminooxy)-, 2R,3S-9-[[1,3-Dihydroxy-4-fluoro-3-butoxy]methyl]guanine, respectively. The results suggest the brown areca nut extract inhibits the anti-inflammatory activity of nitric oxide LPS-stimulated RAW 264.7 cell line. Thus, the regular consumption of areca nut reduces the inflammatory diseases.

Key words : *Areca catechu* L. nut; Phytoconstituents; Antioxidant activity; RAW 264.7 cell line; Anti-inflammatory activity; MTT assay; Lipopolysaccharide

1. Introduction

Areca nut or betel nut is the seed of the fruit areca palm (*Areca catechu* Linn. family Palmaceae), which grows in Asia (India, Bangladesh, Sri Lanka, Myanmar, Taiwan), tropical Pacific, and in some parts of East Africa. It is a common ingredient for many products and approximately 600 million people are widely using it for chew products (13,22). Various parts of the tree (A. catechu) have been used to prepare lubricants, dye-making, preparing pickles, weaves, and fuel wood etc. Traditionally the parts from A. catechu's roots, shoots, leaves, buds, and nuts are appropriately used for healing burn wounds and skin ulcers (6); the users of A. catechu's believe that the plant is also helpful for the supporting the digestive system (7).

The major phytoconstituents of the areca nut are alkaloids, polyphenols, flavonoids, carbohydrates,

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minerals, fats, proteins, and crude fiber (27). Several studies have reported that the secondary metabolites from A. catechu has various biological applications like antioxidant, anti-inflammatory, anti-nematodal, anti-diabetic, hypolipidemic, anti-hypertensive, anti-bacterial, anti-fungal, anti-aging, anti-malarial, anti-viral, anti-HIV, anti-aging, anti-depressant, anti-convulsant, treatment for Alzheimer's, wound healing, anti-ulcer, anti-migraine, anti-hypertensive, anti-depressant, anti-allergic, anthelmintic, aphrodisiac, anti-venom, hepatoprotective, and cytoprotective (16).

Inflammation occurs to eliminate the preliminary cause of cell injury due to the response of the body's protection. Though, sometimes, inflammation it can also become self-perpetuating and cause further inflammation. Chronic inflammation, involved in many diseases, for example, Alzheimer's, dermatitis, ulcerative colitis, inflammatory bowel disease, systemic lupus erythematous, cancer, osteoarthritis, rheumatoid arthritis, and cardiovascular diseases (25).

In the previous study, the hydroalcoholic extract of *Areca catechu* was reported to possess significant anti-inflammatory activity (5). In this context, the present study was planned to evaluate the in vitro anti-inflammatory potency of red and brown *Areca catechu* nuts in murine macrophages raw 264.7 cell lines.

2. Materials and Methods

Collection and extraction of plant material

The Areca catechu red and the brown nut were collected from Dindigul, Tamil Nadu, India, and the collected plant materials were taxonomically authenticated. The red and brown areca nuts were washed under the running tap water to remove dirt and then shade dried. The dried nuts were grinded to obtain coarse powder by using the electrical blender. The powdered materials were stored in an airtight container for further use. 100 g of each plant materials were subjected to Soxhlet extraction using 95 % ethanol for 48 h. The extracts were subsequently filtered through Whatman No.1 filter paper and concentrated to dryness in a vacuum under reduced pressure using a rotary evaporator (20).

Preliminary qualitative phytochemical analysis

Preliminary phytochemical analysis was carried out to identify the phytoconstituents like alkaloids, saponins, tannins, cardiac glycosides, flavonoids, phenols, steroids, terpenoids, quinones, and proteins present in the ethanol extracts of areca nuts red and brown (10).

Test for alkaloids

To the crude extract, 2 ml of Mayer's reagent was added, a dully white precipitate revealed the presence of alkaloids.

Test for saponins

1 ml of the crude extract, 5 ml of water was added, and the tube was shaken vigorously. The formation of stable foam indicated the presence of saponins.

Test for tannins

2 ml of 2% ferric chloride solution was added to the crude extract. Greenish black color indicates the presence of tannins.

Test for cardiac glycosides

1 ml of crude extract, 2 ml of glacial acetic acid containing a drop of ferric chloride. An equal volume of concentrated sulphuric acid was added from the sides of the test tube. A brown color ring indicates the presence of cardiac glycosides.

Test for flavonoids

The crude extract was treated with a 10% sodium hydroxide solution; the formation of intense yellow color indicates the presence of flavonoid.

Test for phenols

The crude extract was mixed with 3 ml of 10% lead acetate solution was added. A bulky white precipitate indicates the presence of phenolic compounds.

Test for steroids

1 ml of crude extract was dissolved in 10 ml of chloroform and an equal volume of concentrated the sulphuric acid was added from sides of the test tube. The upper layer turns red and the sulphuric acid layer showed yellow with green fluorescence indicates the presence of steroids.

Test for terpenoids

1 ml of crude extract was mixed in 2 ml of chloroform, and concentrated sulphuric acid was carefully added to the sides of the test tube. A reddish-brown coloration of the interface indicates the presence of terpenoids.

Test for quinones

The crude extract was treated with an alcoholic potassium hydroxide solution. The appearance of color ranging from red to blue indicates the presence of quinones.

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Test for Proteins

1 ml of crude extract was taken, and few drops of freshly prepared Ninhydrin reagent were added and heated. The appearance of purple color indicates the presence of proteins.

Thin Layer Chromatography (TLC) analysis

A 10 cm long TLC plate silica gel 60 F254 (Merk, Darmstadt, Germany) was cut and marked carefully. 10 mg/ml of stock solution of the samples areca nut red and brown were spotted at about 0.5 cm from the bottom of the plate with the help of a capillary tube. Butanol: acetic acid: water (4 ml: 1 ml: 2 ml) was used as a mobile phase. The TLC plate was then placed in the chamber containing the respective solvent system. After that, the plate was removed from the chamber and air-dried. The plates were then observed under UV light at 254 and 366 nm. The iodine and potassium permanganate reagents were sprayed on the TLC plate and were heated continuously to 100 ºC to visualize the spots. The visible colored spots were marked and the retention factor (Rf) was calculated as follow (4) :

$$R_f = \frac{\text{Distance traveled by substance}}{\text{Distance traveled by the solvent}}$$

Determination of in vitro antioxidant assay

Free radical scavenging assay on DPPH

The DPPH? radical scavenging activity of the crude extracts (Areca nut red and brown) was determined according to the method described by some modification (21). Samples at different concentrations (3.125 - 100 µg/ml) were added to 2 ml solution of DPPH (0.4 mM) in methanol. Ascorbic acid was used as a reference standard. The tubes were shaken and allowed to stand for 30 min at 37°C under dark conditions. The absorbance was measured at 517 nm. % of inhibition was calculated as follow :

$$\% \text{ of inhibition} = \frac{\text{control - sample}}{\text{control}} \times 100$$

ABTS$^+$ scavenging assay

Radical scavenging assay of areca nut red and brown extracts were described by ABTS$^+$ cation decolorization assay (21). ABTS$^+$ was developed by reacting 7 mM ABTS$^+$ aqueous solution with 2.4 mM potassium persulfate in the dark for 16 h at room temperature. Ascorbic acid was used as a reference standard. Samples at different concentrations (3.125 - 100 µg/ml) were added to 1 ml of diluted ABTS solution and mixed thoroughly. The reaction mixture was kept at room temperature in the dark for 30 min, then the absorbance was measured at 734 nm and the percentage of inhibition was calculated as follows (21) :

$$\% \text{ of free radical scavenging} = \frac{\text{control-test sample}}{\text{control}} \times 100$$

In vitro cell culture and maintenance

The murine macrophage RAW 264.7 cell line was purchased from National Centre for Cell Science (NCCS, Pune, India) and maintained in DMEM supplemented with 10% FBS, 100 µg/l streptomycin, and 100 IU/ml penicillin at 37°C in a 5% CO2 atmosphere.

MTT cell viability assay

RAW 264.7 cells were seeded in 96-well plates at the density of 5 x 104 cells/well. After 24 h of incubation, the adhered cells were treated with various concentrations (3.125 - 100 µg/ml) of the extracts. 24 h later, after changing the medium, MTT was added to a final concentration of 5 mg/ml, and the cells were incubated for 4 h at 37°C and 5% CO_2. The medium was then removed, and the formazan crystal was solubilized in DMSO. The absorbance was measured at 570 nm using a microplate reader (14).

Inhibition of nitric oxide (NO) production in LPS-induced Raw 264.7 cell line

The RAW 264.7 cells were seeded at a density of 5 x 104 cells/well in 96-well plate and incubated for 24 h at 37°C and 5% CO_2. Then media of each well were removed and fresh FBS-free DMEM media were replaced. Different concentrations of samples (3.125 - 100 µg/ml) were prepared in FBS-free DMEM to give a total volume of 100 µl in each well of a microtiter plate. After 1 h treatment, cells were stimulated with 1 µg/ml of LPS for 24 h. After, 100 µl of cell culture medium with an equal volume of Griess reagent in a new 96-well plate was incubated at room temperature for 10 min. Then the absorbance was measured at 540 nm in a microplate reader. The amount of nitrite in the media was calculated from sodium nitrite ($NaNO_2$) standard curve (14).

Gas Chromatography-Mass Spectroscopy (GC-MS) analysis

The GC-MS analysis of the potent brown variety of areca nut extract during in vitro anti-inflammatory studies on murine macrophages RAW 264.7 cell line was carried out using a Clarus 500 Perkin - Elmer (Auto system XL) Gas Chromatograph equipped and coupled to a mass

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detector Turbo mass gold - Perkin Elmer Turbomass 5.2 spectrometer with an Elite - 5MS (95% Dimethyl polysiloxane), 30m x 250 m DF of the capillary column. The instrument was set to an initial temperature of 60 °C and maintained at this temperature for 2 min. At the end of this period, the oven temperature was rose up 300°C, at the rate of an increase of 5°C /min, and maintained for 6 min. Injection port temperature was ensured as 240°C and Helium flow rate as one ml/min. The ionization voltage was 70eV. The sample was injected in split mode as 10:1. The mass spectral scan range was set at 40-600 (m/z). Using computer searches on a NIST Version Year 2011 were used GC - MS data library and comparing the spectrum obtained through GC - MS compounds present in the plant sample was identified (15).

Statistical analysis

All the data were expressed as mean ± standard deviation. The data were evaluated with GraphPad Prism 5.01, GraphPad Software Inc. P value less than or equal to 0.05 were considered significant.

3. Results and Discussion

Qualitative phytochemical screening

The present study revealed that ethanol extract of both red and brown varieties of *Areca catechu* nut that contains tannins, cardiac glycosides, flavonoids, phenols, steroids, terpenoids, and proteins. However, the phytoconstituents of alkaloids, saponins, and quinones were not found in both the red and brown areca nut **(Table 1).**

Table 1 : Qualitative phytochemical screening of ethanol extracts of red and brown varieties of *Areca catechu* L. nut

Phytoconstituents	**Ethanol extract**	
	Red areca nut	**Brown areca nut**
Alkaloids	-	-
Saponins	-	-
Tannins	+	+
Cardiac glycosides	+	+
Flavonoids	+	+
Phenols	+	+
Steroids	+	+
Terpenoids	**+**	**+**
Quinones	**-**	**-**
Proteins	**+**	**+**

TLC analysis

Thin-layer chromatography on silica gel revealed that to identify the bioactive molecules from medicinal plants. The secondary metabolites of areca nut (red and brown) extracts were observed under UV light at 254 nm, iodine, and potassium permanganate spraying reagents. The ethanol extract of areca nut red showed six spots with Rf values 0.26, 0.53, 0.66, 0.8, 0.86, and 0.93 respectively while the ethanol extract of areca nut brown showed five spots with Rf values 0.26, 0.53, 0.66, 0.8, and 0.93, respectively **(Figure 1).**

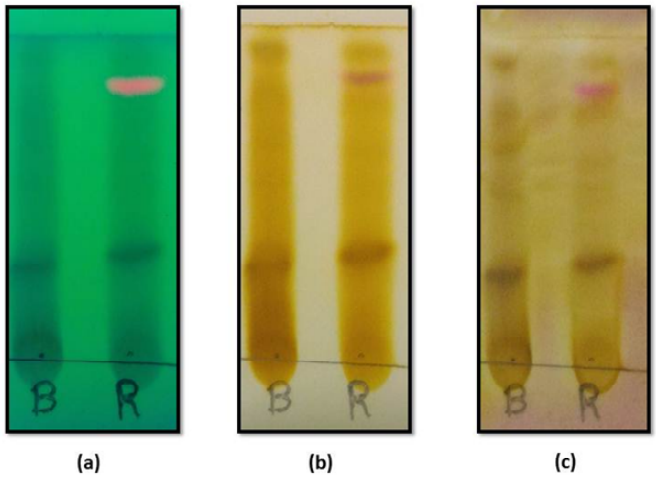

Figure 1 : TLC fingerprint of red and brown varieties of areca nut extracts (a) UV 254 nm; (b) Iodine spraying reagent; (c) Potassium permanganate spraying reagent

In vitro antioxidant activity

DPPH assay

The antioxidant properties of crude ethanol extract from *Areca catechu* nuts (red and brown) were determined using DPPH radical scavenging assay. The results shown in **Table 2** were found to be that the extract of brown areca nut showed better DPPH radical scavenging activity compared with the red areca nut. The mean IC50 values of brown and red areca nut were found in the order to be 24.54 ± 0.14 µg/ml, and 28.26 ± 0.11 µg/ml followed by the standard (ascorbic acid) which showed at 12.14 ± 0.17 µg/ml, respectively.

Table 2 : In vitro antioxidant activity of ethanol extracts of red and brown varieties of *Areca catechu* L.

Antioxidant activity	IC_{50} values of samples (µg/ml)		
	Red areca nut	Brown areca nut	Ascorbic acid
DPPH	28.26 ± 0.21	24.54 ± 0.14	12.14 ± 0.07
ABTS	32.24 ± 0.01	26.55 ± 0.08	6.10 ± 0.01

*Data represented as mean ± Standard deviation (n=3); statistically significant level at ($p<0.05$).

$ABTS^+$ assay

The IC50 values of $ABTS^+$ scavenging assay of red and brown areca nut extracts are shown in Table 2. The

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most effective of brown areca nut and least activity of red nut were found to be 26.55 ± 0.08 µg/ml, 32.24 ± 0.01 µg/ml, and the standard ascorbic acid showed 6.10 ± 0.01 µg/ml, respectively.

In vitro cell line assay

Ethanol extracts of red and brown areca nut on Raw 264.7 cell viability assay

The ethanol extracts of Areca catechu nut (red and brown) against Raw 264.7 cell line was screened by MTT assay. The IC50 values of both extracts red and brown areca nut were found to be >100 µg/ml, respectively **(Figure 2).**

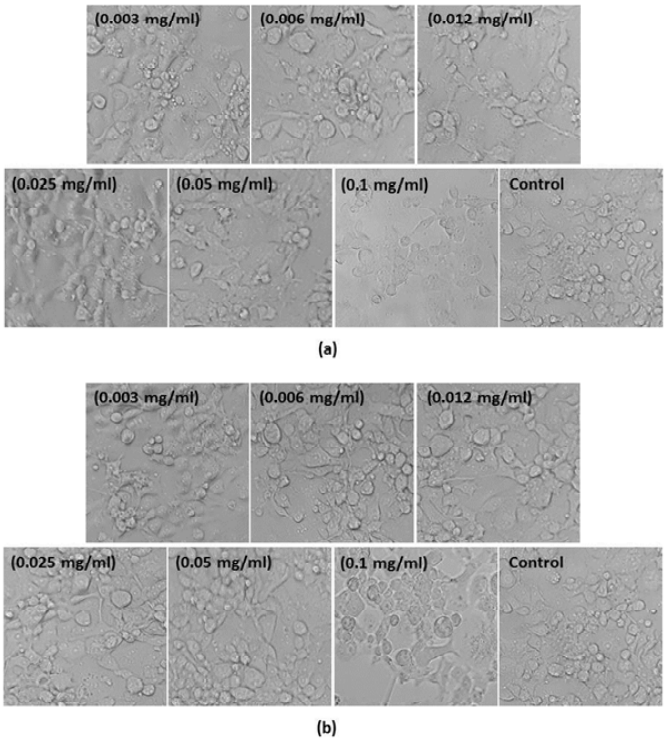

Figure 2 : Different concentration of areca nut (red and brown) extracts on RAW 264.7 cell line. (a) Represents red areca nut extract treated on RAW 264.7 cell line. (b) Represents brown areca nut extract treated on RAW 264.7 cell line

Inhibition of nitric oxide production in LPS-stimulated Raw 264.7 cell line

In figure 3 represents there is no nitrite could be detected in the untreated Raw 264.7 cells. Once the cells were stimulated with LPS, the increased level of nitrite was produced. The brown areca nut inhibited nitric oxide formation more than red areca nut. Various concentrations (3.125 - 100 µg/ml) of brown and red areca nut extracts shows the percentage of nitrite level as follow (81.22, 69.26, 60.53, 33.59, 13.72 and1.95 nitric oxide/ml, and 84.52, 73.74, 65.26, 38.5, 16.92 and 3.41nitric oxide/ml), respectively.

GC-MS analysis

The Gas Chromatography-Mass Spectroscopy (GC-MS) of the brown areca nut extract of chromatogram shows in Figure 4. The identified secondary metabolites follow (2S,3S) - (-) -3-Propyloxiranemethanol, Propanedioic acid, propyl, 1,1-Dodecanediol, diacetate, 2-Nonenoic acid, Carbromal, 3-Nonenoic acid, Pentanoic acid, 2-(Aminooxy)-, 2R,3S-9-[[1,3-Dihydroxy-4-fluoro-3-butoxy]methyl]guanine, respectively **(Table 3).**

The secondary metabolites of plant-derived chemical compounds are important for drug development from pharmaceutical industries (2, 23, 24). Tannins, alkaloids, and flavonoids compounds were found to be present from the ethanol extract of areca nut (23). Acetone extract of both the leaves and root showed the presence of saponins and tannins. Root extract also showed the alkaloids, carbohydrates, terpenoids, and sterols. The phytoconstituents has antimicrobial activity which developed the zone of inhibition against bacteria viz., *Klebsiella pneumonia*, *Staphylococcus epidermidis, Bacillus subtilis, Enterococcus faecalis* and *Enterobacter aerogenes*, and fungus viz., *Aspergillus fumigatus, Aspergillus niger, Candida albicans, Candida glabrata,* and *Candida tropicalis* (1). The methanol extract of areca nut shows tannins, glycosides, steroids, phenols, resins, carbohydrates, and quinones. The presence of bioactive components was reported strongly active against protein denaturation assay (11). The phenolic compounds were identified from water, methanol, and DMSO extract from areca nut shows the Rf value 0.73, respectively (17).

Antioxidant activity plays an important role in therapeutic applications. It prevents oxidative stress and control the various diseases from humans (26). Anthikat and Michael, (2012) investigated the aqueous extract of areca nut shows the DPPH and ABTS radical scavenging assay of IC50 value found to be 95 µg/ml, and ABTS assay found to be 9.5 µg/ml, respectively (3). The author investigated methanol extract from the areca nut showed the IC50 value of 0.021 µg/ml, respectively (9).

During inflammation, macrophages play an important role in the body against pathogens (8). The secondary metabolites of *Areca catechu* are pharmacologically important for several therapeutic purposes especially in treating inflammatory conditions. In the present study, the MTT assay showed the cell viability of areca nut (red

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and brown) extracts have no toxicity against RAW 264.7 cells. The maximum concentration (100 µg/ml) of samples showed the IC50 value ranging from > 100 µg/ml, respectively. In this LPS-induced nitric oxide production assay in RAW 264.7 cell line, the various concentrations of brown areca nut significantly decreased the nitric oxide content than red areca nut extract. Similarly, it has been reported that ethanol extract from areca leaf inhibits the NO levels against RAW 264.7 cell line (18). The natural compound linalool shows anti-inflammatory activity against the RAW 264.7 cells (12). The extraction of hawthorn fruit aqueous fraction significantly decreases the NO production in RAW 264.7 cells (19).

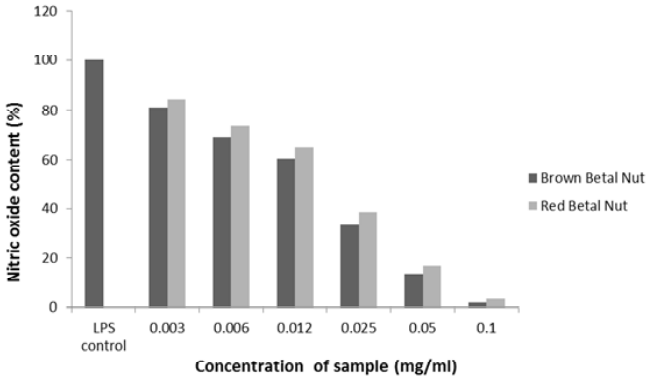

Figure 3 : Inhibitory effect of red and brown varieties of ethanol extract of areca nut on nitric oxide production in a culture medium of LPS-stimulated RAW 264.7 cell lin *Data represented as mean±Standard deviation (n=3); statistical significant level at ($p<0.05$).

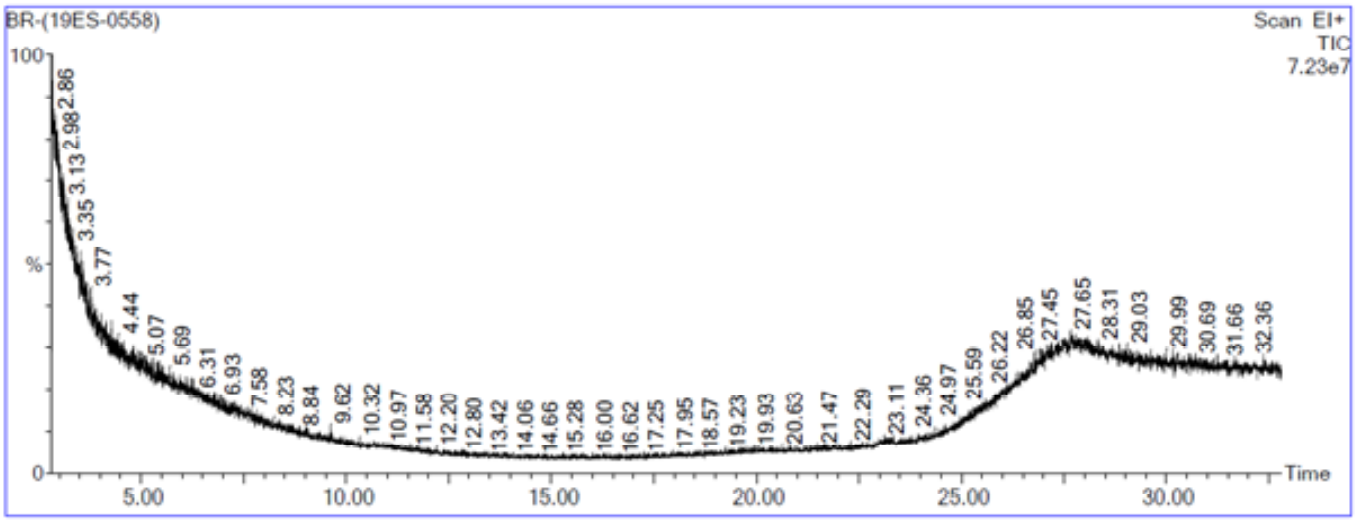

Figure 4 : GC-MS chromatogram of ethanol extract of brown areca nut

Table 3 : Compounds identified in the ethanol extract of brown areca nut

RT	Name of the compound	Molecular formula	Molecular weight
27.073	[(2S,3S)-3-propyloxiran-2-yl] methanol	$C_6H_{12}O_2$	116.16
27.368	Propanedioic acid, propyl	$C_6H_{10}O_4$	146.14
27.483	1,1-Dodecanediol, diacetate	$C_{16}H_{30}O_4$	286.41
27.603	2-Nonenoic acid	$C_9H_{16}O_2$	156.22
28.074	Carbromal	$C_7H_{13}BrN_2O_2$	237.09
28.314	3-Nonenoic acid	$C_9H_{16}O_2$	156.22
28.424	Pentanoic acid, 2-Aminooxy)-	$C_6H_{13}NO_3$	147.17
28.674	2R,3S-9-[[1,3-Dihydroxy-4-fluoro-3-butoxy] methyl] guanine	$C_{10}H_{14}FN_5O_4$	287.25

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4. Conclusion

The conclusion of this present study indicates that the brown areca nut from ethanol extract has more effective in reducing the level of nitric oxide in lipopolysaccharide (LPS)-stimulated RAW 264.7 cells than red areca nut extract. The given evidence from GC-MS shows the brown areca nut extract comprises the compound guanidine that supports the treatment of inflammatory diseases.

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Current Trends in Biotechnology and Pharmacy 19
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Antioxidant and Antihyperlipidemic Activity of Methanolic Fraction of *Maytenus heyneana* Root on STZ Induced Diabetic Wistar Rats

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Abstract

In earlier in-vitro anti diabetic and anti oxidant study with serial fractionation of *Maytenus heyneana* root, indicated methanol fraction as the most effective.In the present investigation, methanol fraction wasfurther tested for in vivo antioxidant and hyperlipidemic activity on STZ induced diabetic rats. Secondary metabolites of MFMH wereidentified by phytochemical screening. Single dose interperitoneal injection of STZ (45 mg/kg) was used to induce the hyperglycemia. To confirm the hyperglycemia, blood glucose was measured,where as hyperglycemia induced oxidation was the determined by using enzymatic (SOD & CAT), non enzymatic (GSH) antioxidants and oxidative stress parameter was evaluated by LPO (TBARS). To access the antihyperlipidemic activity, the TC, TG, HDL-C and LDL-C were measured. Histology of liver was screened. Phytochemical studies revealed the presence of alkaloids, saponins, flavonoids, phenols, cardiac glycosides and terpenoids. Treatment with the methanolic fraction of *Maytenus heyneana* was effective in reducing the blood glucose level and also found to be potent antioxidant by significantly increase SOD, CAT & GSH and significant decrease in oxidative stress LPO. The dose dependent MFMHon antihyperlipidemicactivity was found, by ameliorating the increasedlevel of TC, TG, LDL-C and increased the level of HDL-C. Degenerated hepatocytes of STZ diabetic rats were restored to normal morphological features as like reference standard glibenclamide. Hence from observation, the MFMH possesses antioxidant as well as antihyperlidimic activity on STZ induced diabetic rats.

Key words : *Maytenus heyneana* root, Antioxidant, Antihyperlipidemic activity, Methanolic fraction, STZ diabetic rats

1. Introduction

At least 68 % of individual's age of 65 or more with diabetes dies from some type of coronary illness; and 16% dies of stroke. Grown-ups with diabetes are two to multiple times more likely to die from coronary illness than Grown-ups without diabetes (1). The American Heart Association considers diabetes to be one of the seven significant controllable risk factors for cardiovascular disease. There are afew factors that play important role in pathogenesis of diabetes namelyoxidative stress and hyperlipidemia leading to higher risk of complications (2). Oxidative stress (ROS) owe to the prolonged hyperglycemia is possibly the onset and development of type 1 & II diabetes and its complications such as stroke, retinopathy, nephropathy and neuropathy and also itdevelop the vascular complications in diabetes particularly type 2 diabetes. In diabetes, oxidative stress is brought about by advanced glycation end products (AGEs) and free radical development via autoxidation of unsaturated lipids in plasma and membrane proteins. It might be amplified & propagated by an autocatalytic cycle of metabolic stress, tissue damage, and cell death leading to a concurrent increment in free radical production and compromised inhibitory and scavenger mechanisms, which further exacerbate the oxidative stress (3).

Oxidative stress in diabetes is coincides with a decrease in the antioxidant power. Diabetes tends to decrease "good" cholesterol levels (HDL-c) and increase triglyceride and "bad" cholesterol levels (LDL-c), which builds the risk for heart disease and stroke, a condition called Diabetic dyslipidemia (4). Hyperlipidemia associated with diabetes is a medical as well as social problem, leading to increasing both morbidity and mortality. Hyperglycemia & hypercholesterolemia were related with oxidative change of LDL-C, protein

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glycation, glucose -autoxidation.The significant objective of diabetes treatment is to keep blood glucose, lipid protein and lipids levels near to normal and scavenge ordetoxify the free radicals. As of late, drug formulation from natural plants, for treatment of diabetes mellitus and other diseases, fascinated the attention of many scientists (5). Since various studies have demonstrated that hyperglycaemia in diabetes mellitus commits to oxidative stress, it has been suggested that the constant supply of natural products as an antioxidants might reduce the oxidative stress, and hence protect harmful effects of oxidation stress on tissues (6, 7, 8).

On account of strong antioxidant properties; during recent years many species of plants have been studied for the management and treatment of various diseases. For this reason, research work is being conducted to suggest an approach that involves certain agents tending to alleviate the hyperglycemia induced oxidative stress and hyperlipidemia. Genus of Maytenus, aflowering and fruiting plants consists of about 300 species and it is widely distributed in dry deciduous forests in tropical and subtropical areas and it has a wide range of pharmacological actions such as antibacterial, antileukemia, gastroprotective, anti-inflammatory, antiulcer. analgesic, antinociceptive, antidiarrheal (9), anti-malarial (10), anti amobic dysentery, anti-tuberculosis (11). *Maytenus heyneana* **(Fig 1)** armed shrubs, synonym of Gymnosporia heyneana belongs to Celastraceae family distributed mainly southern parts (Tamil Nadu, Kerala, Andhra Pradesh and Karnataka) of India. It has been used as antiarthritic, antidiabetic, anti-snake venom, immunity boosters, cancerous wound healing, and antidysentery (12). Research shows that bark of *Maytenus senegalensis*, leaf of *Maytenus emarginatus* and root of *Maytenus putterkloides* found to possess antidiabetic activity. *Maytenus senegalensis* and *Maytenus royleanus* have reported to possess an antioxidant effects.

In vitro antidiabetic and antioxidant activities of differentsolvent fractions of *M. heyneana* root have been recorded in our previous studies (12). Furthermore, we have observed significant increase in free radical scavenging activity against DPPH and ABTS and inhibitory action against α-amylase and β-glucosidase with the methanol fraction of *M. heyneana* root (12). Based on the in vitro anti diabetic and antioxidant properties of *M. heyneana*, the current experiment was designed to evaluate the protective effects of methanol fraction of *M. heyneana* (MFMH) root against the STZ induced oxidative stress and hyperlipidemia in diabetic wistar rats.

2. Materials and Methods

Chemicals and reagents

All the chemicals and reagents used were of analytical grade. Streptozotocin (STZ) was purchased from Sigma (Sigma-Aldrich Ltd., Mumbai, India). Glibenclamide (GLA) was gifted from Hetro Pharm, Vishakpatnam.

Preparation of samples

The selected plant fraction was evaporated to remove the solvent and concentrated powder was used for *in-vivo* evaluation. The plant fractions were designated as MFMH (methanolic fraction *Maytenus heyneana*). The dried fraction powder were dissolved in saline solution and givento animals by orally.

Toxicity studies and selection of the dose

The acute toxicity studies was performed for the MFMHaccording to OECD-423 (Organization of Economic and Cooperation Development), fixed dose guidelines. All the experiments on animals were designed and conducted in accordance to ethical norms approved by (Institutional animal ethical committee) IAEC (NCP/ IAEC/2016-17-17). Generally, healthy adult albino female rats of Wistar strain (n=3) were used. The animals were starved for 3 hours with free access to water only. Single oral doses of MFMH were administered with a preliminary dose of 200mg/kg body weight (b.w). The animals were monitored for mortality for 3 days. If mortality was not ascertained the procedure is repeated with higher dose (2000 mg/kg) of the selected plant fractions. General behaviors (13) was also observed during the acute toxicity study such as Hypnotics, Sedative, Convulsion, Motor activity Ptosis, Analgesia, Stupar reaction, Muscle relaxant, Pilo erection, Changes in skin color, Stool Consistency and Lachrymal secretion.

Animals

Healthy, adult male albino wistar rats were selected for the study. They were housed in clean polypropylene cages under standard laboratory conditions with temperature around 26 °C. The rats were acclimatized to the laboratory conditions prior to any treatment for 10 days prior to any treatment and had ad libitum access the food and water on a 12:12 day/night cycle. Throughout the experiment cage bedding of animals was changed frequently following the STZ induction of diabetes due to polyuria

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Induction of Diabetes

Diabetes was provoked in male rats by intraperitoneal injection (i.p) of 45 mg/kg of STZ (Sigma Aldrich Chemical Co, St. Louis, MO, United states of America (USA) reconstituted in 0.1 M cold citrate buffer (pH 4.5) after an overnight fast. The glucose levels were determined in blood collected by tail vein puncture, 72 hrs after STZ administration, utilizing Accu-check Advantage II clinical glucometer (Roche Diagnostics Co.,). Fasting blood Rats with glucose 250 mg/dl were considered diabetic and were included in the study

Study Design for in vivo Studies

To assess the antioxidant and antihyperlipidemic activity of the MFMH following 5 groups were made and six animals for each group **(Table 1).**

Table 1. Depicts Study Design for in vivo studies

Group 1	Normal control rats were given normal saline (5 ml/kg/b.w) orally
Group 2	Diabetic rats were given normal saline (5 ml/kg/b.w)orally
Group 3	Diabetic rats were given MFMH (*Maytenus heyneana* methanol fraction) 200mg/kg dissolved in normal salineorally
Group 4	Diabetic rats were given MFMH (*Maytenus heyneana* methanol fraction) 400mg/kg dissolved in normal salineorally
Group 5	Diabetic rats given Glibenclamide (GLB) at 10 mg/kg dissolved in 0.5% Carboxy methyl cellulose (CMC)orally

The MFMH, Glibenclamide were administered by orally to their respective group of rats up to 14 days. During the study the rats were fed with 10% w/v glucose solution, in their cages in order to avoid the diabetic shock. The blood was collected from tail of the rats. The glucose level was measured by glucometer (Accu Chek). At the 14th day (end of the study period), all the animals were anaesthetized with diethyl ether and the blood samples were obtained by puncture of retro-orbital plexus using glass capillary (20mm) and stored with or without ethylenediaminetetraacetic acid (EDTA) (2mg/ml) to evaluate the lipid profile and antioxidant parameters. Then, they were sacrificed and theliver was collected immediately after dissection and stored at -20 ^{o}C for further histopathological studies and small portion of liver was dissected for tissue homogenate preparation.

Serumpreparation

The collected blood without EDTA was allowed to clot by keeping undisturbed in a room temperature for 30 min. The clot was removed by centrifuging for 10 min at 4000 rotation per minute (rpm) in a refrigerated centrifuge (REMI, INDIA). The supernatant serum was collected and used for serum lipid profile analysis.

Preparation of liverhomogenate

The liver was rapidly removed and perfuse immediately with ice-cold 0.9% sodium chloride (NaCl). A portion of the liver was homogenized in chilled Tris-HCl buffer (0.025 M, pH 7.5) using a homogenizer (REMI, Bombay, India) with a Teflon plunger. The homogenate thus obtained was centrifuged at 5000 rpm for 10 min. The supernatant was collected and used for oxidative stress parameter (Lipid peroxidation-TBARS method).

Hemolysate preparation for antioxidant activities

Hemolysate was prepared by McCord and Fridovich et al., proposed method (14). 5 ml of blood sample was collected in an EDTA bulb, 2 ml was separated for estimation of GSH and the remaining sample is centrifuged at 2000 rpm for 10 min. The plasma was then removed. The Red blood cell (RBCs) was washed twice with normal saline. Nearly 3.5 ml of purified water was added to the RBCs. This hemolysate was utilized for catalase estimation. Then the HB (hemoglobin) concentration of the remaining hemolysate was measured. The concentration of HB was adjusted to 10gm/dl. To 0.5ml of Hb adjusted hemolysate, 3.5 ml of ice-cold water (distilled water) was added and mixed well on a cyclomixer until all the particulate matter disappears. To this solution, 1 ml of ethanol was added and again mixed on cyclomixer. Finally, added 0.6ml of chloroform and mixed well. Then this hemolysate was centrifuged for 10 min at 3000 rpm. The clear supernatant was used to estimate the superoxide dismutaseactivity.

Estimation of blood glucose and antioxidant parameters

The blood was collected from tail vein at 0, 7th and 14th day and the level of blood glucosewere monitoring using Accu-chek advantage glucometer and Roche glucostrip (15).

Enzymatic antioxidant reduced glutathione (GSH) in blood was estimatedspectrophotometrically by Beutler et al 1983 (16). Non enzymatic antioxidant such as Superoxide dismutase (SOD) in hemolysate was estimated by the method of Marklund and marklund

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1974 (17) and the catalase (CAT) in hemolysate assay was estimated by the method of Aebi 1983 (18). The oxidative stress parameter Lipid peroxidation (LPO) in liver homogenate was estimated colorimetrically by TBARS (thiobarbituric acid reactive substance) by the method of Niehaus and Samuelsson 1968 (19).

Estimation of lipid profile parameters

The total serum concentration of cholesterol wasdetermined by enzymatic CHOD-PAP method (Allainet al.,1974) (20) using commercially available biochemical kits (Recon Diagnostics). The total serum triglyceride was estimated by GPO-PAP (Bucolo G and Harold D 1973) (21) using commercially available biochemical kits (Recon Diagnostics).The serum HDL-C was estimated by precipitation method and followed by CHOD-PAP method described by Burstein et al., 1970(22). The serum LDL-Cand VLDL-C was estimated by calculation using the empirical relationships according to Friedewald's formula (Friedwald et al., 1972) (23).

Preparation of liver for histopathologicalstudies

The whole isolated liver was washed in saline solution and 10% formalin was used for fixing and paraffin for embedding and sections were cut of 3-5 m thickness and stained with haematoxylin & eosin are basic dye and acidic dye respectively. The sections were microscopical studied at 10× and 40× magnifications for the islet cell characteristics using a binocular compound microscope.

Statisticalanalysis

In animal study, data were denoted as mean ± Standard Error Mean (SEM). The statistical analysis was done by using Analysis of Variance (ANOVA), followed by Dunnett's test for multiple comparisons using Graph Pad Prism (version 5) software and values of Probability Value (P)< 0.05 were considered as statistically significant.

3. Results and Discussion

Diabetes mellitus is a chronicmetabolic disorder that has arguably achieved epidemic proportions. It affects more than 371 million people globally, and itis projected to affect 522 millionpersons by the year of 2030 (24) (25) (26). For some decades, phyto-therapy has played asignificantrole in the treatment of the disease particularly in poor resource countries. Clearly, theidentification of plant materials that can treat the diabetes mellitusand its complications would seven million of people life, particularly in developing countries, from untimely death. From our earlier reported studies showed the presence of phytoconstituents such as saponins, phenols, terpenoids, flavonoids and tanninsin the MFMH root may contribute to its hypoglycemic activity (12), and these compounds have been shown to beresponsible for hypoglycemic activity in Momordica charantia (27). Presence of flavonoids and phenols in MFMH could be a reason for scavenge active oxygen species and effectively prevent from oxidative cell damage (12). Our results are confirmed with those announced by Ramachandran V and Saravanan R 2013 (28).

Acute toxicity studies

WHO recommends that medicinal plants would be dominant source to obtain a wide range of drugs. Therefore, medicinal herbs must be investigated for its better understanding of their pharmacological activity, effectiveness and safety and well involves the recognition of a dose level that causes mortality (29).Thus, acute toxicity studies of MFMH was performed on adult albino female rats of wistar strain were used because of minor difference in sensitivity between sexes; therefore females rats were commonly used for its more sensitiveness (30) to finding out the quantitative aspect of lethal dose (LD50) and observing the behavioral studies. From the results (Table 2) it was observed that MFMH did not shown any sign of toxicity, mortality or a dying status in a female rat. The LD50 of MFMH roots was observed to be greater than the test dose (2000mg/kg). Therefore, low dose of 200mg/kg b.w and high dose of 400 mg/kg b.w were used for our studies.

Hyperglycemic activity

Hyperglycemia was induced in male rats, because female rats are less susceptible to streptozotocin at high doses, this decreased susceptibility of females may be presence of estradiol, which able to protect the oxidative stress induced pancreatic β cells apoptosis (31) the single dose of IP injection of 45 mg/kg of STZ reconstituted in the 0.1 M cold citrate buffer at pH, in order to prevent the degradation and maintain the stability of STZ (32). After administration STZ, triphasic response of blood glucose was produced. Initially raise of blood glucose due to breakdown of liver glycogen, secondly persistent hypoglycemia and it was more pronounced in fasted rats, which may cause early mortalities, 10% glucose solution has given to experimental rats to prevent from mortality. Finally, permanent hyperglycemia was observed (33). This STZ hyperglycemia was might be due to cytotoxic effect of on GLUT2 receptor present in β cell membrane, liver and kidney, this cytotoxic action is more pronounced

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on β cell membranes, which in turn to triggers the multiple pathways such as advanced glycation end (AGE) product, hexosamine pathway, polyol pathway, protein Kinase C pathway, and poly adipose ribose polymerase pathway all pathways contribute towards oxidative stress by generation of ROS in mitochondria. The results disclosed that single dose STZ is highly effective cytotoxic agent for pancreatic β cells and complications in liver, kidney and other vital organs such as heart, brain and muscles (34). STZ - diabetes has been described as a useful experimental model to study the antidiabetic activity of several agents (35).Glibenclamide the reference standard is like other sulphonylureas, is effective against in mild diabetic condition, but ineffective against severe diabetic condition, where the β cells were destroyed completely (34).

The change in level of blood glucose of control and experimental groups of diabetic rats were observed are represented graphically in Fig 2. On the day 0, the level of blood glucose was significantly raised (P<0.001), compared to control group. Remarkably (P<0.001) increase in blood glucose level on 7th day was noticed as 288.83 mg/dl on diabetic control group, when compared with normal control group. However, treatment given to diabetic rat with MFMH at the dose 200 & 400 mg/kg, the level of blood glucose was significantly (P<0.001) decreased to 234.50 & 184.67 mg/dl respectively. In diabetic group, the level of blood glucose was further significantly increased (P<0.001) to 308.67 mg/dl at the last of 14th day. When treated STZ diabetic rats with MFMH at the dose 200 mg/kg, the level of blood glucose was significantly (P<0.001)declinedto179.33mg/dl and it was further significantly turned down to 147.50 mg/dl with the case of treated STZ diabetic rats with MFMH at a dose 400 mg/kg. Thus the antidiabetic action of MFMH may act directly by stimulating the insulin secretion in existing active β cells. Further, the fundamental antihyperglycemic activity of MFMH was associated with increase in plasma insulin level and or insulin like extra pancreatic action of reduction of hepatic gluconeogenesis & glycogenolysis and with increase utilization of glucose by peripheral tissue. Our results are in confirmed with those announced by Raju Patil et al., (36).

Antioxidant activity

Table 3. Illustrates that the oxidatative stress LPO level in liver homogenate, enzymatic antioxidant superoxide dismutase, catalasein hemolysateand non-enzymatic antioxidant Glutathione inwhole blood.

The oxidatative stress LPO in diabetic group shows significant increases (P<0.001) from 11.61 to 24.06 nM/mg. Whereas, the diabetic groups with MFMH treatment at different doses of 200 and 400 mg/kg b.w decreases the LPO to 19.60 and 13.34 nM/mg respectively, which are statistical significance of p<0.001, compared with the diabetic group.

Diabetic group shows enzymatic antioxidant superoxide dismutase was significantly declined (P<0.001) to 9.89 IU/mg compared to that of the normal control group 20.30 nM/mg. However, the SOD in hemolysate was significantly raised (P<0.001) to 12.89 &15.15 nM/mg in groups with MFMH treatment at the concentrations of 200, 400 mg/kg of body weight respectively. Whereas, catalase (CAT) is pronounceable dropped (P<0.001) to 14.86 nM/mg, when it is compared to the normal group 27.87 nM/mg. The levels of CAT in hemosylate is observed significantly increased (P<0.001) to 21.47 & 24.36 nM/mg in the diabetic animal groups treats with MFMH at the dose of 200, 400 mg/kg respectively. In case of non-enzymatic level of antioxidant glutathione (GSH) was significantly (P<0.001) decreased to 31.45 nM/mg, compared to normal group (52.07 nM / mg). The glutathione in hemolysate was significantly increased (P<0.001) to 37.89 and 46.41 nM /mg in the diabetic groups treatment with MFMH at the concentrations of 200, 400 mg/kg respectively. Also noticed from the results that effect of MFMH (400mg) on LPO,CAT,SOD & GSH exhibits identical to standard glibenclamide and normal group.

Chronic hyperglycemia can generates oxidative stress, which gives rise to cellular tissue damage through important five mechanisms (37). The highly complexed antioxidant systems in the human body (enzymatic and non-enzymatic), which work together synergistically to defend the cells and organs against free radical damage.STZ acts selectively on β-cells of pancreas lead to increase ROS in pancreas & other tissues are resulted in tissue damage & increases LPO i.e., membrane lipid oxidation. Increase LPO is an indication of decreasing defense mechanisms of both enzymatic and non-enzymatic antioxidants (38). MDA has reported as a primary biomarker of the free radical mediated lipid damage and oxidative stress. The elevated level of MDA in an erythrocytes ofthe diabetic rats was observed, this could be a reason of ROS mediated chain propagation reaction might be lost activity of defense antioxidant system to adequately scavenge the free radicals produced by STZ induced diabetes. Conversely, decreased in the

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serum LPO level and MDA was monitored in MFMH and glibenclamide treated diabetic animals, this perhaps by the inhibition of the propagation chain reaction of LPO (39). Additionally, possible way of oxidative stress in hyperglycemia also may by the account of an autoxidation of glucose, redox imbalances, reduced concentration of LMW antioxidants, such as GSH (reduced glutathione) and reduced activities of antioxidant defense enzymes such as SOD (superoxide dismutase) and CAT (catalase) (40). The enzymatic antioxidant (GSH) and the non-enzymatic antioxidants (SOD, CAT) are the cellular defense mechanism that acts on the free radical by removal of hydroxyl, hydrogen peroxide and superoxide radicals. SOD is the key enzyme in detoxifying the superoxide radical and converts into hydrogen peroxide and water. H2O2 is highly reactive small molecule which formed as results of the energy metabolism of natural product which in an excessive level may cause appreciableamount damages to RNA, DNA, lipids, and proteins (41). CAT is the main regulator of H_2O_2 metabolism and which enzymatically neutralizes it, thus protects the pancreatic β cells from hydroxyl radicals' attack (42) (43). The diabetic groups shown decrease in erythrosylate SOD and CAT concentration. This might be the cause of an excess production of superoxide radicals which inhibit the activity of the SOD and CAT activities (44), which consequently enhanced after treatment with MFMH and glibenclamide, be a sign of strife against ROS generation. GSH act as reducing agent, which detoxify the H_2O_2 in the existence of glutathione peroxidase (GPx) enzyme (44).The current study shows decreased in concentration of erythrocytes GSH in diabetic control, this could be because of increased scavengingactivity in the repair of free radicals caused biological damage. Reduction in antioxidant capacity with increased oxidative stress could be related to the complications in patients with diabetes like oxidative DNA damage and insulin resistance (45) due to reduced antioxidant potential of blood, diabetes complications increase which include nephropathy, cardiovascular disease, nerve damage and blindness. Thus, the increasing the occurrence of diabetes is a remarkable health concern beyond the disease itself (46). Whereas, MFMH and glibenclamide treatments restore the GSH concentration, maybe by reason of the up regulation of GSH redox system in liver to counteract oxidative stress. The treatment and MFMH also observed declines in the level of LPO and elevated concentrations of SOD and CAT in diabetic animals as equal as reference standard glibenclamide. A similar finding has been reported on the study with Mangiferin (41).

Lipid profile assessment

Fig 3. Represents effect of leaves of EAFOG and MFMH root on total cholesterol, triglycerides, HDL-c &LDL-C. The level of cholesterol was significantly high ($P<0.001$) from 91.14 to 145.28 mg/dl while in diabetic animals, the cholesterol have significantly ($P<0.05$) reduced to 130.58 by the treatment EAFOG at the dose of 200mg/kg. Similarly significant declined ($P<0.001$) in cholesterol (110.28 mg/dl) in Streptozotocin induced diabetic group treated with extracts MFMH at the dose level of 400 mg/kg.

Whereas, the serum triglyceride levels was significantly ($P<0.001$) increased to 146.11 mg/dl in diabetic animals. However, there is significantly ($P<0.001$) reduced to 111.42 and 105.19 mg/dl while in treatment with MFMH at different doses of 200 and 400 g/kg respectively. In case of serum HDL-c level is seen significantly declined ($P<0.001$) to 26.56 mg/dl in the diabetic group. In the case of diabetic control animals, which were fraction of EAFOG at the dose of 200, 400 mg/kg are observed in increases upto 37.24 & 38.31 mg/dl respectively. The results of HDL-c showed, significantly increased ($P<0.001$) to 96.90 mg/L, when it is compared with normal group. The increased in LDL-c level is significantly lowered ($P<0.001$) to 86.61 and 66.01 mg/dl by MFMH at the dese level of 200 and 400 mg/kg respectively.

Dyslipidemia is a well known complication of diabetes (47) and accompany with hyperglycemia characterized by increased LDL-c, increased triglycerides (TGs) and decreased cholesterol. In present study, the elevated serum cholesterol, triglycerides and LDL-c while decreased levels of HDL-c was observed in Streptozotocin induced diabetic rats, this observation also conforms with reports of earlier studies (48) In diabetic induced rats, the glucose level is increased together with the lipids level shows that the insulin dependent tissue plays main role in glucose and lipid homeostasis (49). Insulin resistance initiates the intracellular hormone-sensitive lipase, which in turn to increases the release of NEFA (non-esterified fatty acid) from triglycerides in adipose tissue (50); the raised NEFA consecutively increases the formation of hepatictriglyceride. The NEFA is converted to cholesterol and phospholipids when combining hepatic triglyceride and released as in form of lipoproteins into blood stream. Hyperlipidaemia was produced in Streptozotocin diabetic

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group might be regarded as the result of uninhibited actions of lipolytic action of hormones in the fat storage (51). LDL-c oxidation positively and HDL-C dysfunction negatively contributes to risk of cardiovascular disease (52). The increased TG in the diabetic groups was observed that may be chance as to the increased the absorption & production of TG in form of chylomicrons and decreased uptake of TG by peripheral tissues. The elevated total cholesterol may be because of increased small intestione absorption of cholesterol (53). Whereas, treatment with MFMH at different doses of 200 and 400 mg/kg of b.w have not just decrease the total cholesterol, TG and LDL-c but also increases the HDL-c, HDL-c plays a key role in transport of peripheral tissue cholesterol back to liver by the pathway termed as "reverse cholesterol transport' which consider to be a cardio protective lipid. A highly negative relationship between HDL-c and the occurrence of atherosclerosis are reported and our findings are in an agreement with earlier studies of Poonam S et al., (54). Interestingly, MFMH treated groups shown significant development in the lipid profile comparable to glibenclamide in the control of hyperglycemia (diabetic dyslipidaemia) this might be due to presence of active metabolites. This hypolipidemic effect could represents the protective mechanism against atherosclerosis development.

Assessmentof histopathology of liver

Histopathology of liver revealed the existence of normal histological structure, regular distinct hepatocytes with sinusoidal spaces arranged radically around the central vein, normal hepatic parenchyma with normal portal triads and sinusoids in normal control group. In diabetic group, the section liver shows that hepatocytes degeneration, cytoplasm vacuolization, cloudy swelling, loss of glycogen granule and congested central vein. A hemorrhagic focus has replacing necrotic hepatocytes. Infiltration of leucocytes in portal triads is observed. Also visualization of Kupffer cells are hypertrophied along with hepatic sinusoids, hepatocytes with various degree of degeneration. Treatment with MFMH at the low dose of 200 mg/kg shows Leucocytic infiltration in portal triad and thickening of the portal artery wall. Kupffer cells are Hypertrophied also seen along with the sinusoids and partially distorted architecture, leucocytic infiltration and proliferation of bile canaliculi is also visualizes. In case of high dose of MFMH 400 mg/kg shows, shows mild inflammatory infiltration in periportal region. There are scattered inflammatory cells amidst these hepatocytes. The veins in the central are appears and few sinusoids are seen dilated (Fig 4). The GLB treated liver section shows, recovery of liver structure and normal granule of glycogen contents present in hepatocytes. Liver parenchyma shows intact architecture. The perivenular hepatocytes and mid zonal hepatocytesappears.

As the sensitive organ liver have a great capability to detoxify the toxic substances, excrete the xenobiotic and its metabolitesand also plays vital roles in carbohydrate metabolism (55). Consequently, the pathological damages that are impose on the liver by hepatotoxic agents show to be fatal in diabetic conditions. The liver is most frequently damaged organ during diabetes, as a consequence of increased oxidative stress, which is generated by free radicals and dysregulation of immune function (56) (57). Additionally long term insulin resistance and impaired insulin secretion contribute the deterioration of diabetes by impairment in mitochondria of islet β cells (58) along with mitochondrial dysfunction of insulin-sensitive tissues such as muscle,liver and heart (59). Hepatocytes degeneration, cytoplasm vacuolization, congested central vein and hypertrophied Kupffer cells were noticed in diabetic group.Interestingly these severely damaged pathological conditions which were seen in diabeticrats are restored almost to its normal liver morphological features by MFMH and glibenclamide, which depicts evidence of cellular regeneration. These results are echoed by the earlier published reports (60) (61).

4. Conclusion

Overall, the administration of MFMH led to lower the ameliorated hyperglycaemia and promotedthe correction of dyslipidemic activity in the treatment groups. In addition, MFMH increases the level of SOD, CAT and GSH- activity and reduced level of LPO activity and consequently could alleviate complications of liver this might be due to the improvement of glycaemia promoted by MFMH. The imbalance between the generation of ROS and enzyme activity be controlled and also protect against the development of atherosclerosis on diabetic rats.Hence, it may be concluded that MFMH root at 400 mg/kg b.w exhibited promising antioxidant and hyperlipdimic activity in STZ-induced diabetic rats. However, Further studies is needed to isolate the active components of MFMH.

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Fig 1. (a) Moderate-sized armed shrub with terminating short shoots (b) Exhibits large dominant taproot.

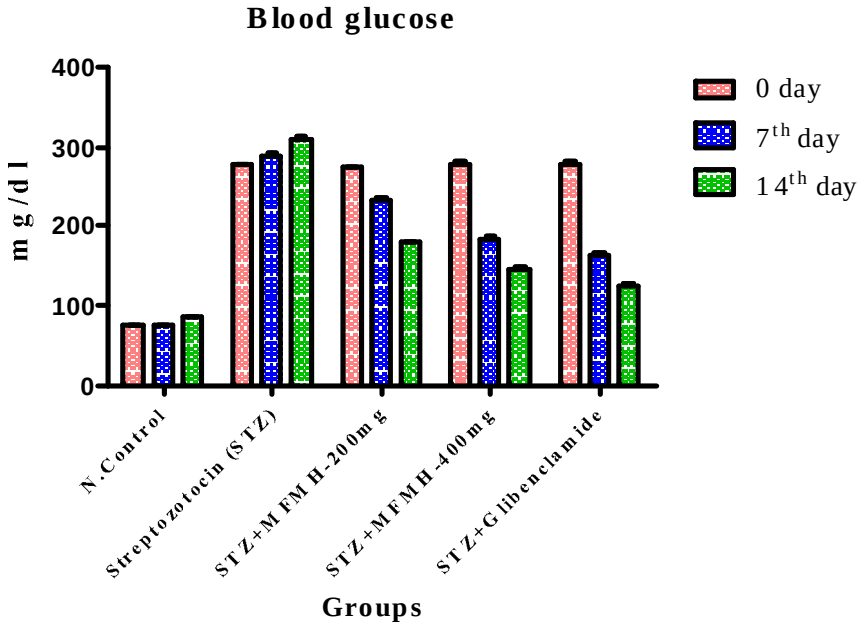

Fig 2. Effect of leaves of MFMH root on the level of Blood Glucose

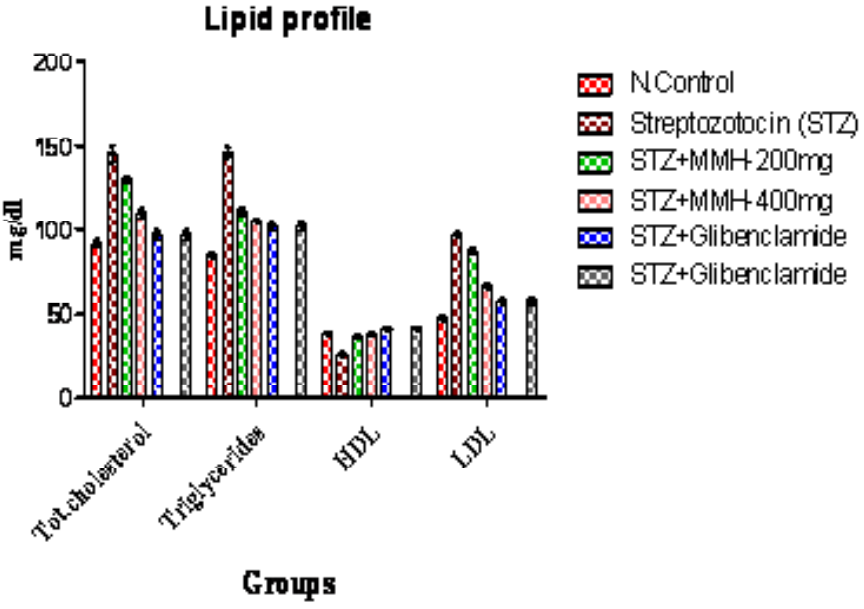

Fig 3. Represents effect of MFMH root on total cholesterol, triglycerides, HDL-c &LDL-C

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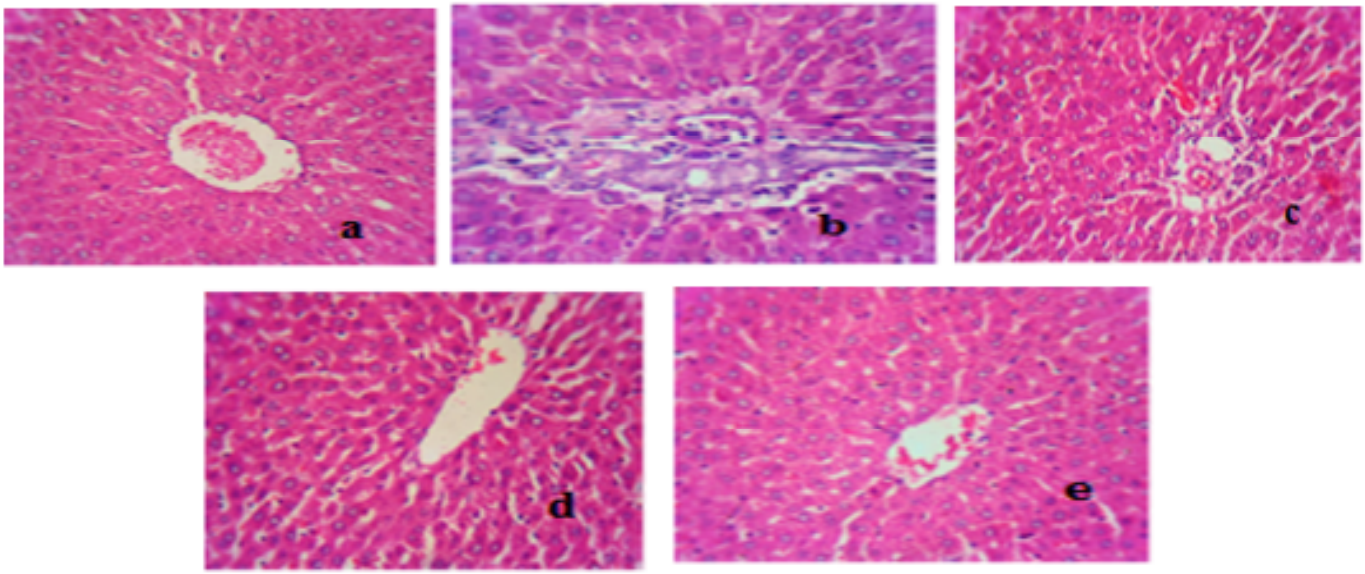

Fig 4. (a) : Normal control rats, (b) STZ induced diabetic control rats,
(c) Diabetic rats treated with MFMH 200mg/kg (d) Diabetic rats treated with MFMH 400mg/kg.
(e) Diabetic rats treated with reference standard glibenclamide 10mg/kg

Table 2. Specifies the observation of behavioral studies of EAFOG and MFMH on rats

General Behaviors	**Time (hrs)**					
	1	**2**	**3**	**12**	**24**	**72**
Hypnotics	-	-	-	-	-	-
Sedative	-	-	-	-	-	-
Convulsion	-	-	-	-	-	-
Motor activity	-	-	-	-	-	-
Ptosis	-	-	-	-	-	-
Analgesia	-	-	-	-	-	-
Stupar reaction	-	-	-	-	-	-
Muscle relaxant	-	-	-	-	-	-
Pilo erection	-	-	-	-	-	-
Change in skin color	-	-	-	-	-	-
Stool Consistency	-	-	-	-	-	-
Lacrimal secretion	-	-	-	-	-	-

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Table 3. Effect of EAFOG and MFMH on Antioxidant parameters SOD, CAT, GSH & Oxidatative stress parameter LPO

Groups	**Antioxidant parameters**			**Oxidative stress parameter**
	SOD (IU/mg of protein)	**CAT (nM of H2O2 decomposed/ min/mg of protein)**	**GSH (nM /mg of protein)**	**LPO (nM of MDA/mg of protein)**
Group I Normal control Normal saline 10ml/kg	20.30±0.41	27.87±0.24	52.07±0.99	11.61±0.99
Group II Diabetic control STZ 45mg/kg	9.89±0.34 a***	14.86±0.48 a***	31.45±0.69 a***	24.06±0.69 a***
Group III STZ +MFMH-200mg/kg	12.89±0.24 b***	21.47±0.66 b***	37.89±0.25 b***	19.60±0.25 b***
Group IV STZ +MFMH-400mg/kg	15.15±0.39 b***	24.36±0.25 b***	46.41±0.50 b***	13.34±0.50 b***
GroupV STZ +GLB-10mg/kg	19.59±0.75 b***	26.68±0.75 b***	50.41±0.89 b***	11.85±0.89 b***

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Development and Assessment of Modified Glover Nilsson Vaping Behavioural Questionnaire Among Malaysian Electronic Cigarettes Users

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Abstract

The Glover Nilsson smoking Behavioural Questionnaire (GNSBQ) is the commonly used scale to assess the behavioural nicotine dependence through conventional tobacco cigarettes (TCG). But the GNSBQ does not evaluate the subject's behavioural dependence to nicotine that administered via electronic cigarette (EC). The study aim was to develop and assess an equivalent modified Glover Nilsson vaping behavioural questionnaire (GNVBQ) which measures the nicotine behavioural dependency via EC. The modified developed GNVBQ scale is identical to the original GNSBQ. The scale scores indicate the EC nicotine behavioural dependency ranking as slight (1-6), mild (7-11), moderate (12-22), strong (23-33) and very strong (> 33). The scale piloted among 15 EC single users i.e. used only EC. The assessment of the scale did among 69 EC single users and observed their nicotine behavioural dependency status for a one-year period. The modified scale revealed a satisfactory Cronbach's alpha value of 0.74. Further test-retest reliability of the scale showed an acceptable spearman's rank correlation coefficient value of 0.75 ($p > 0.05$). A one-year observation showed that out of 69 EC single users, 11 single users completely stopped nicotine intake. The EC users who completely stopped nicotine intake after one year had a low nicotine behavioural dependency scores between 7-11 measured by the modified GNVBQ scale. The modified GNVBQ scale has accurately identified the behavioural dependence of nicotine that administered via EC. Therefore, as per the current study results, the modified GNVBQ scale may useful to predict the nicotine behavioural dependency that administered through various EC products. However, the scale needs to be validated further with a large sample size for further robust authentication.

Key words : Electronic cigarette, Nicotine, Vaping, Behaviour, Dependency, Scale

1. Introduction

Nicotine dependence is a complicated experience that encompasses both physiological and behavioural components [1]. The diagnostic and statistical manual of mental disorders edition 5 (DSM-5) stated that tobacco use disorder is assigned to individuals who are dependent on the drug nicotine. According to DSM-5, there are 11 possible criteria for nicotine dependence of which at least 2 must exist in the last 12 months among smokers: 1)Tolerance, indicated by increased amounts of tobacco to achieve the desired effect or a markedly diminished effect with continued use of the same amount of tobacco 2) Withdrawal symptoms, shown by either the characteristic withdrawal syndrome or the use of tobacco to relieve the withdrawal symptoms. 3) Craving or urge to use tobacco 4) Tobacco has taken in larger amounts or over longer periods of time 5) Persistent desire or unsuccessful efforts to cut down nicotine 6) Spending much time to obtain tobacco 7) Recurrent tobacco use resulting in a failure to fulfil the major role obligations at work, school, or home 8) Continued tobacco uses despite having persistent or interpersonal problems caused e.g., disputes with others for tobacco use 9) Important social, occupational, or recreational activities are given up or reduced because of tobacco use 10) Recurrent of tobacco use in situations in which it is physically hazardous 11) Tobacco use is continued despite knowledge of having a persistent or psychological problem that likely to cause by tobacco use.

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The criteria from 1 to 3 such as tolerance, withdrawal and craving show the physiological dependence to nicotine. Whereas other characteristics signify the behavioural dependence to nicotine. The physical dependence on nicotine is measured by various scales. The most applied scales are Fagerstrom test for nicotine dependence (FTND) [2], nicotine dependence syndrome scale (NDSS) [3] and Wisconsin inventory of smoking dependence motives (WISDM-68)[4]. The world health organisation (WHO) international classification of diseases-10 also assess the physical dependence on nicotine [5]. However, none of the earlier mention scales addresses the nicotine behavioural element of dependence [6]. The behavioural component of nicotine addiction is demonstrated through the smoker's patterns of tobacco use such as smoking style, opening the cigarette pack, pulling a cigarette out of the pack, hand to mouth cigarette action, smoking along with some daily activities like drinking coffee or driving a car. The behavioural pattern of smoking includes the cognitive, social, and behavioural effects correlated with nicotine dependence. Smoking cessation products can fulfil the physical dependence to nicotine but not yet replaced the behaviour involved with nicotine dependency [7].

It has well documented that smokers are physically and behaviourally dependent on nicotine. Many earlier studies have shown that smoking cessations medications like nicotine replacement therapy, varenicline, and bupropion along with behavioural and motivational counselling showed a good quitting rate as compared to medication alone [8-9]. The FTND scale used extensively in the smoking-related research to assess the physical dependence to nicotine, whereas behavioural dependence on smoking measured by the Glover-Nilsson smoking behavioural questionnaire (GNSBQ) [10]. The GNSBQ scale exchange smoker and cigarette relationship related to feeling any rituals associated with smoking. The GNSBQ consists of 11 questions and responses to the questions on a 0-4 scale, where 0=not at all, 1=somewhat, 2=moderately, 3=very much and 4=extremely. GNSBQ upper score indicates higher behavioural dependence on smoking, whereas lower numerical shows low behavioural dependence. Scoring for GNSBQ follows ranking as mild (1-11), moderate (12-22), strong (23-33) and very strong (> 33). The smokers who score more on GNSBQ scale may treat suitable through behavioural counselling[11]. However, a smoker revealed higher score on FTND display high physical dependence to nicotine, which can manage well by pharmacological interventions. If a smoker shows, the high score on both the scales requires both behavioural counselling and pharmacological treatment.

Literature findings revealed lacking the scale that measures the behavioural dependence to nicotine that administered through electronic cigarette (EC). The GNSBQ measured the nicotine dependence via tobacco cigarettes (TCG) but do not point out the subject's behavioural dependency to nicotine by using EC. There are various nicotine EC products in the markets with distinct concentration [12-13]. Therefore, it is a necessity to assess the behavioural dependency of nicotine by means of EC for consumer's safety and public awareness purposes. In order to treat effectively the nicotine dependency among vapers, it is suitable to recognize both behavioural as well as physical dependence to nicotine via EC. Thus, the current study developed a scale by modifying the existing GNSBQ scale which predicts the behavioural dependence of nicotine that administered through the various EC products.

2. Materials and Methods

Scale development

The scale was modified from GNSBQ [10]. The modified scale scores like original GNSBQ which consists of 11 questions and measures the nicotine behavioural dependence using EC. The scale has a total score of 44. Each question is ranked on a 0-4 scale. The values in scale described as 0=, not at all, 1=somewhat, 2=moderately, 3=very much and 4=extremely respectively. The modified Glover-Nilsson vaping behavioural questionnaire (GNVBQ) explained about vapers relationship related to feeling, perception and any rituals associated with EC. Higher the score, more behavioural nicotine dependence to EC, whereas lower case shows low nicotine behavioural dependence via EC. Scoring for behavioural dependence follows ranking as slight (1-6), mild (7-11), moderate (12-22), strong (23-33) and very strong (> 33). The modified scale has a variation as compared to the original GNSBQ scale. The principal change is replacing the word tobacco cigarette with an electronic cigarette.

Validity and Reliability of the scale

The developed scale was sent to 5 experts in associated disciplines to evaluate the face and content validity. The reviewer individually ranked each item of the scale by using a four-point grading system i.e. 1=not relevant, 2= somewhat relevant, 3=relevant, 4=highly relevant. The

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Item Content Validity Index (I-CVI) was applied to assess the validity of each item. The items on the scale with CVIs ranged from 0.80 to 1.00 had retained. The calculated average I-CVI scale value was 0.95 with average content validity ratio (CVR) of 0.99 [14]. The Face validity of the scale was also assessed in terms of feasibility, readability, clarity and uniformity of the language. The face validity was determined on a 1-4 scale which denoted as 4=strongly agree, 3=agree, 2=disagree, and 1 strongly disagree. All experts were graded three or four on the scale and indicated the questionnaire is feasible and appeared understandable to the desired population. The items and scoring guide of the modified developed scale has shown in table 1.

Table 1: Modified Glover Nilsson Vaping Behavioural Questionnaire

Scale: 0=Not at all, 1= somewhat; 2=moderately ; 3=Very much; 4=Extremely	
How much do you value the following (Specific to Questions 1-2).	
1. My vaping habit is very important to me	0 1 2 3 4
2. I handle and manipulate my electronic cigarette (EC) as part of the ritual of vaping	0 1 2 3 4
Please indicate your choice by circling the number that best reflects your choice. (Specific to Questions 3-11). **Scale: 0=never; 1=seldom; 2=sometimes; 3=often; 4=Always**	
3. Do you place something in your mouth to distract you from vaping?	0 1 2 3 4
4. Do you reward yourself with vaping after accomplishing a task	0 1 2 3 4
5. If you find yourself without vaping, will you have difficulties in concentrating before attempting a task?	0 1 2 3 4
6. If you are not allowed to vape in certain places, do you then play with your EC?	0 1 2 3 4
7. Do certain environmental cues trigger your vaping, e.g., favourite chair, sofa, room, car, or drinking alcohol	0 1 2 3 4
8. Do you find yourself vape routinely without craving?	0 1 2 3 4
9. Do you find yourself placing an EC in off mode in your mouth and sucking to get relief from stress, tension or frustration, etc	0 1 2 3 4
10. Does part of your enjoyment of vaping come from the steps while switching on your EC?	0 1 2 3 4
11. When you are alone in a restaurant, bus terminal, party, etc., do you feel safe, secure, or more confident if you are holding an EC?	0 1 2 3 4
Total	
Scoring 1-6 : Slight ; 7-11 : Mild ; 12-22 : Moderate; 23-33 : Strong ; > 33 : Very Strong	

Pilot study

The modified developed scale was piloted among 15 EC single users i.e. who use only EC verified by exhaled carbon monoxide (CO) of < 8 ppm. The internal consistency of the scale was determined by using Cronbach's alpha which revealed a satisfactory value of 0.74. Moreover, the reliability of the scale was further accomplished by the test-retest method by followed up among all 15 users of the pilot study after a period of two weeks interval. The reliability of the scale after two-week intervals showed a spearman's rank correlation coefficient value of 0.75 with $p > 0.05$. The P-value indicated that there was no significant variation at two different interval periods and the scale is stable over time. Finally, the approved piloted scale was tested among 69 EC single users and observed their behavioural dependence of nicotine that administered through EC.

Assessment of the developed scale

The study participants were enrolled from EC sales points, vaping stations surrounding at the Kuantan and Pekan districts, province Pahang, Malaysia by distributing flyer related to the study. The study partakers have contacted the researcher and clarified any queries related to the study before the enrolment process. The information sheet and consent forms were given to the

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committed participants. The participants who met the eligibility criteria were selected for the enrolment process. The socio-demographic details, history of smoking packs per years, and EC were reported. In the previous study, the investigator assesses the modified FTND scale to determine the physical dependence to nicotine that administered via EC. In the current study, the researcher among the same cohort assessed the behavioural dependency to nicotine by modified GNVBQ scale. The participants were observed for a one-year period. At week 52, all the subjects were verified through the biochemical validation by measuring the CO test irrespective of any smoking status. At week 52, self-reported complete nicotine quit participants additionally evaluated by the saliva NicAlert® strips to check their full nicotine-free status. At week 52, subjects without biochemical validation were documented as nicotine users for analysis purpose. Intention-to-Treat (ITT) analysis was applied to evaluate the final outcomes of the study. That means those users who lost to follow-up were categorised as nicotine users. Also, participants who withdrew from the study were omitted for analysis.

Ethical Committee Approval

The study was approved by the research ethics committee (IREC) of Kulliyyah of Medicine, International Islamic University of Malaysia (IIUM) Kuantan on 9th October 2014, with IREC registration number 302. The study was also registered in the National Medical Research Registration with NMRR.number:15-180-24825.

3. Results and Discussion

After a one-year observation period out of 69 EC single users, 11 EC single users were completely stopped nicotine intake. The other 24 remained as EC single users, 15 shifted to dual use i.e. using both EC and TCG and 19 relapsed to TCG. All study participants nicotine status validated by CO level and saliva cotinine tests respectively. Those EC single users who completely stopped nicotine intake after the one-year period had a low nicotine behavioural dependency score of 7-11 measured by modified GNVBQ scale. Figure 1 shown participants behavioural nicotine dependency score measured by modified GNVBQ scale at baseline and their nicotine status at week 52.

The current study results demonstrated that the modified GNVBQ is a reliable and valid scale to assess the nicotine behavioural dependence that administered via EC. The developed scale has good internal consistency shown by Cronbach alfa value of 0.74 and the reliability value of 0.75 respectively. The developed scale revealed that the participants who had low behavioural dependence stopped completely the nicotine intake. The GNVBQ can be considered as a predictive scale of behavioural nicotine dependence. The previous studies indicated that nicotine users who had high behavioural dependence have more desire for nicotine as compared to low nicotine users, irrespective of their physical dependence. The modified GNVBQ scale accurately assess the desire for nicotine via EC by the behavioural gesture. It is believed that the desire for nicotine is the most challenging symptoms of nicotine withdrawal that may hinder vapers to completely stop nicotine intake via various EC products.

The current study findings also suggested that the use of nicotine via EC induced behavioural nicotine dependency. The current study results revealed that at the end of week 52 most of the participants were still using nicotine. The study supports the notion that nicotine plays a vital role in the popularity of EC use [13]. The current study also showed that the use of ECs even for an extended period was unable to crack the nicotine addiction. It is also possible that most of the study participants were using ECs as an alternative device for nicotine. The current study findings are comparable with previous literature studies which too indicated that administration of nicotine via vaping will not induce complete nicotine cessation [15-16]. Therefore, there is a possibility that vaping may uphold nicotine addiction due to availability of e-liquids in various flavours and might renormalize smoking behaviour among vapers. Therefore, tobacco control policy makers should restrict the sale of nicotine-containing EC to non-smokers and youngsters to prevent its abuse. Most of the study participants believed that EC is a worthy choice to curb smoking because it does not induce physical or behavioural dependency. Though, the current study results failed to establish the spotless capability of EC in complete nicotine cessation as compared to other FDA-approved smoking managements medications trials [17-18].

The current study results indicated that it is necessary to identify nicotine behavioural dependence among vapers in addition to physical nicotine dependence. Since medication therapy along with behavioural and motivational counselling revealed a satisfactory nicotine cessation results in previous literature studies [8-9]. Therefore, more customized nicotine cessation treatment

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is needed in vapers based on their nicotine physical vs behavioural dependency.

The current study is not without limitations. The current study was conducted among small vapers population in just two locations of Malaysia i.e. Pekan and Kuantan. Therefore, the scale needs to verify on a large sample size before applying in any EC related studies. Further, factor analysis and construct validities are needed to achieve for robust authentication of scale. Moreover, the existing study population mainly consists of male even though our enrolment was not aimed at male vapers. Therefore, it would be difficult to generalize these results to female vapers regardless of their intention to quit. In addition, more clinical trials are needed to verify the concept of nicotine behaviourally dependent. This is required to determine the exact treatment needed for nicotine-dependent users either by behavioural intervention or pharmacological or a combination of both.

4. Conclusion

The modified GNVBQ scale may precisely identify the behavioural dependence to nicotine which administered via EC. Therefore, the modified GNVBQ scale can apply to predict the behavioural dependence on nicotine that administered through various EC products. However, the scale needs to be validated further with a large sample size for further authentication.

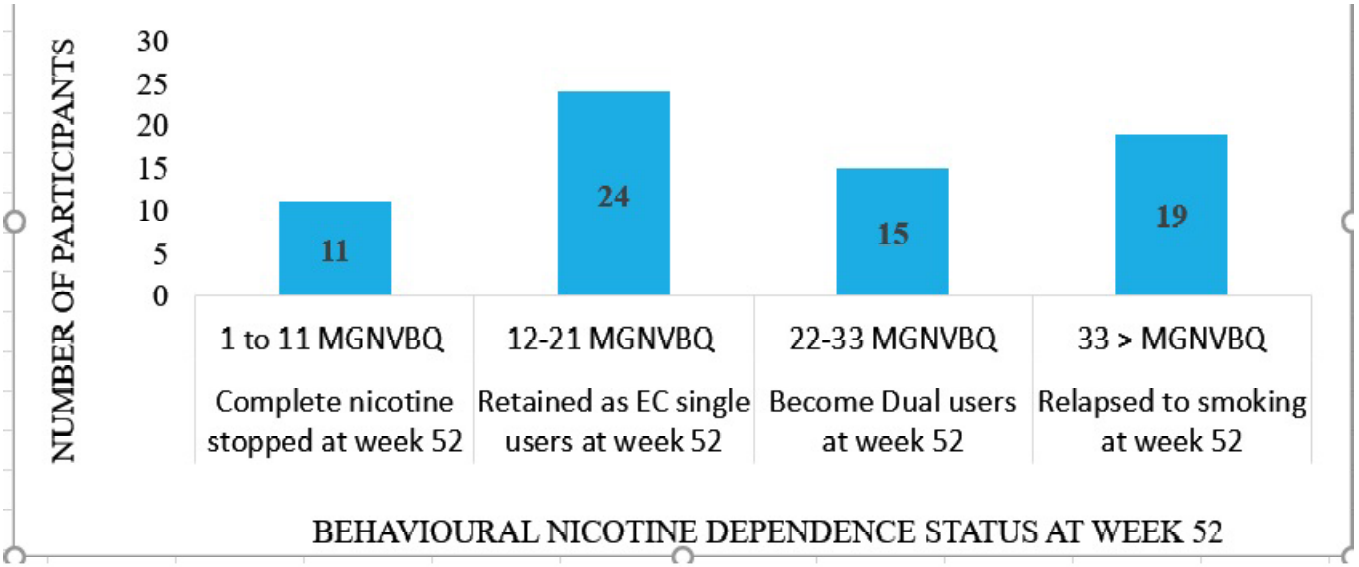

Figure 1 : participants EC behavioural nicotine dependence measured by modified Glover-Nilsson Vaping behavioural questionnaire (MGNVBQ) scale at baseline and their nicotine status at week 52.

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ATR-FTIR Spectroscopy Methods for Determination of Aminoglycoside Antibiotics in Ophthalmic and Parenteral Preparations with Full Partial Least Squares Algorithm

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Abstract

Attenuate Total Reflectance-Fourier Transform Infrared (ATR-FTIR) Spectroscopy methods were developed and validated for the analysis of selected aminoglycoside antibiotics (AGAs) as per ICH guidelines. This non-destructive technique was utilized as a direct measurement of sample after grinding to prepare the powder required priorto read on diamond reader of ATR-FTIR spectrophotometer. Dilutions were made using spectroscopic grade potassium bromide (KBr) on %w/w basis.Linear concentration ranges from 0.25 - 15.0 (%w/w)were observed with high regression value (r2> 0.995)on their unique peak bands using full spectrum partial least squares (PLS)algorithm. These methods weredisplayed low limit of detection (LOD) of 0.20-0.25 (%w/w) and low limit of quantification (LOQ) of 0.60-0.80 (%w/w) for three selected AGAs (Gentamicin, Tobramycin and Kanamycin). The intra-day and inter-day precision values were found with %RSD less than 4.00.Assay studies were shown withtheir mean recovery estimated at 100.727 ± 2.65% for gentamicin, 101.04±1.076% for tobramycin, and 100.67% ±1.08%at 95% confidence intervals for the quantification of gentamicin and tobramycin in ophthalmic preparations and kanamycin in injections. These methods were found to be simple, faster, non-destructive with full sample recovery and eco-friendly. Hence, proposed ATR-FTIR spectroscopy methods can be used for routine analysis and samples approval for regulatory and therapeutic drug monitoring studies during treatment with AGAs with good precision and accuracy comparatively with conventional LC-MS methods, which are time consuming and complex method parameters and also needs dedicated space, funding, manpower and time consuming for immediate results.

Keywords : ATR-FTIR Spectroscopy, Aminoglycoside antibiotics, estimation, validation, non-destructive technique, injections and ophthalmic preparations

1. Introduction

AGAs are potent broad-spectrum bactericidal agent that are particularly active against aerobic, gram-negative bacteria and act synergistically against certain gram-positive bacteria well. Other than being antibacterial agent for human health, the AGAs are widely used in veterinary medicine and agriculture as well (1-3). They are chemically characterized by two or more amino sugars linked by glycosidic bonds to an aminocyclitol component. The cyclitol is 2-deoxystreptamine in most of the AGAs, one exception being streptomycin, which has streptidin moiety. Figure 1 portrays the main chemical moiety and addition of different functional groups at active sites in different types of AGAs.

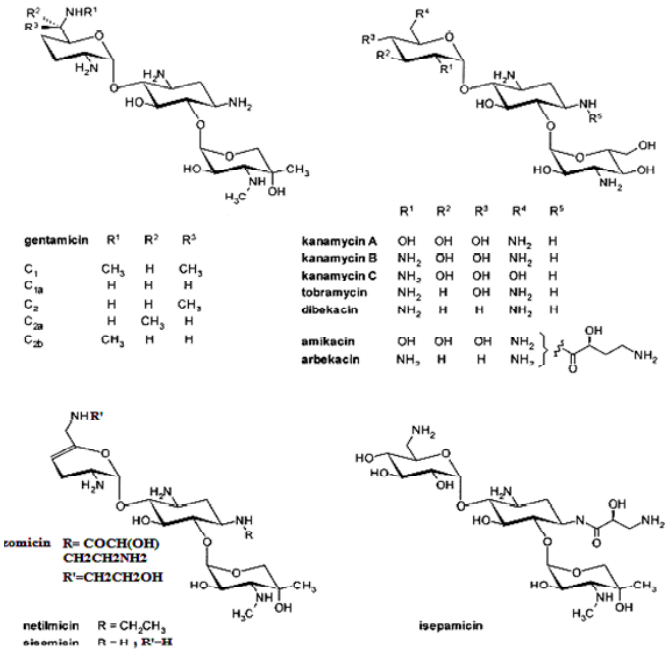

Figure 1. Chemical Structures of AGAs

The selected AGAs that will be tested in this study are gentamicin, tobramycin and kanamycin. Gentamicin consists of three different closely related aminoglycoside sulphates, Gentamicin C1, C2, and C1a, obtained from

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Micromonosporapurpurea and related species, with molecular formula C21H43N5O7 and average 477.60g/mol molecular weight(4).According to United State Pharmacopoeia (USP) 39, the content of gentamicin C1 is between 25-50%; the content of gentamicin C1a is between 10-35%; and the sum of content of gentamicin C2a and C2 is between 25-55%. Slight variation in percentage was claimed by European Pharmacopeia(5-7). On the other hand, tobramycin is a broad-spectrum antibiotic produced by Streptomyces temerarious which is effective against gram-negative bacteria, especially the pseudomonas species. Its molecular formula is C18H37N5O9 with molecular weight of 467.51g/mol(8).

For the determination of gentamicin and its impurities, USP 39 and European Pharmacopoeia 6.8. (Ph. Eur. 6.8.) recommended ion pairing HPLC with pulsed amperometry detection (HPLC-PAD), whilemicro biological assay is described for quantitative analysis. The USP monograph of kanamycin and quantification in formulation also utilize HPLC-PAD for assay (5,9-11).The method of pre-column derivatization with 2,4-dinitrofluorobenzene followed by reversed-phase HPLC with UV detection is described in USP for tobramycin analysis (12).The reported methods for analysis for the selected AGAs cannot be used for routine analysis due to its complex method parameters and lack of UV chromophore. Also, expensive pH-stable column with the need of frequent maintenance for good column efficiency is a factor to be considered as well (10,13,14). Microbial assay requires 24-72 hours incubation time, and is subject to variability (agar thickness, inoculums concentration, incubation temperature, exposure-time duration) and biological error (15). Therefore, development and validation of a good, fast, sensitive and economical analytical method is crucial for the routine quality assessment.

The non-destructive attenuated total reflectance Fourier transform infrared (ATR-FTIR) spectroscopy is rapidlygaining popularity in the development of alternative methodology for the quantification of pharmaceutical drug for its suitability in commercialization. The environmental-friendly method also reduces the usage of hazardous chemical reagent (16). Therefore it is considered as green analytical chemistry. Diamond sample reader facilitates the analysis of all sample state and directly provide refined spectra without sample destruction. PLS regression algorithm is a commonly used multivariate calibration method in baseline correction which utilizes TQ software integrated with OMNIC software to assist users in easy calibration and manipulation of spectra(17). It is widely applied in ingredient identification, grade verification, content uniformity and concentration prediction.

The direct ATR-FTIR spectroscopic study was conducted for standard gentamicin and tobramycin with their respective pharmaceutical formulation available in local pharmacy, which is ophthalmic solution. In the proposed method, the specific functional groupsof gentamicin, tobramycin and kanamycinthat represent for its identification and quantification were explored with PLS algorithms of good linear regression coefficient. Drying process of the ophthalmic preparation and injection sampleswas optimized to ensure the samples in solid form after drying process. All method parameters were set and followed by ICH guidelines of analytical method development(18).ATR-FTIR spectroscopy methods were statistically compared with independent t-test to observe the sensitivity among AGAs. Direct ATR-FTIR methods were simple, fast, non-destructive and accurate. determination of selected AGAs.

2. Materials and Methods

Materials and reagents

Gentamicin, tobramycin and kanamycin sulphate standard were purchased from Sigma-Aldrich company (USA); IR spectroscopic grade potassium bromide (KBr)99.999%, and HPLC grade ethanol 95% were purchased from Merck company (Darmstadt, Germany). Ophthalmic solution (Beagenta eye/ear drops 0.3% w/v formulation in 5mL bottle, and Tobrex solution 0.3% w/v formulation in 5mL) were procured from local pharmacies in Kuala Lumpur, while Meiji kanamycin IM injection formulation in 3mL was procured fromSomedico Pharma companyto be used for quantification determination in compliance with the claimed amount in the formulation.

Instrumentation

Infrared spectra were obtained with NicoletTM iS5 FTIR spectrometer (Thermo Scientific, Madison, Wisconsin, USA) with iD5 ATR accessory featuring a diamond crystal. The spectra were collected against the diamond window background controlled by OMNIC software for spectra collection and TQ Analyst software for data processing (Thermo Scientific, USA). The instrument is equipped with iD5 ATR accessory featuring a top plate diamond crystal with a fixed angle of incidence of 42^{o}.

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ATR-FTIR method development

All spectra were recorded at 4cm^{-1} resolution with average of 20 scans in the range of 4000-600cm^{-1}. In addition, a background scan of air was applied prior each sample scan. An analytical balance with 0.01 mg readability and minimum weight of 2mg in fine range was used. All measurements were taken at ambient temperature and samples were stored in a desiccator when not in use.

AGA standards were diluted with IR gradeKBr to prepare a series of concentration of drug standard ranging 0.25% to 15% w/w. Different amounts of selected AGAs were weighted and mixed with proportionate amount of pure KBr, to get a total weight of 0.1 g of each working standard. The mixture of KBr and gentamicin standard was done in a dry mortar till homogenization reached. The mixture was then transferred to Eppendorf tube to be mixed using vortex for 5 minutes. Each working standard was applied on the ATR-FTIR instrument to scan for its spectrum within the range of 4000 to 650cm^{-1}. A background scan of air was done prior to each scan of working standard. All spectra were recorded at 4cm^{-1} resolution with average of 20 scans per spectrum. The diamond crystal of the ATR accessory was cleaned by Ethanol 95% intermittently between each scan. The working standards were used to observe the standard calibration curve with points ranging from 0.25-15% w/ w. From OMNIC and TQ Analyst software, two types of region including peak area and fixed height location were recorded for identification of suitable peak to generate calibration curve with R2 value higher than 0.995. The measured data of standards and calculated standards were used for linear regression analysis.

Validation of ATR-FTIR methods

According to ICH guidelines, validation criteria included are inter-day and intra-day precision, accuracy, linearity and sensitivity. All samples spectra were tested following the above stated criteria. For linearity, nine serial dilutions of AGA working standards with low concentration ranging 0.25-15% w/w were used to generate a calibration curve in PLS algorithm by scanning from 4000-650cm^{-1}. The sensitivity of method was determined from calibration curve with respect to limit of detection (LOD) and limit of quantification (LOQ). For the determination of LOD and LOQ, selected single spectrum band was measured at lowest concentration of AGA standards, and repeated three times to obtain substantial signal. Precision criteria was evaluated by preparing three different concentrations of standard across the concentration range and analysis of each concentration was repeated three time at different time point. Intra-day repeatability and inter-day precision were measured and recorded in terms of relative standard deviation (%RSD). Quantification of AGA formulation was also done based on calibration curve. The samples were dried into powder form in Binder RE53 Gravity Convection Oven at 60 ^{o}c to remove water in the sample and vortexed equally before direct analysis of the powder. The powder was spiked with 0.4, 1 and 5% w/w of AGA standards respectively. The FTIR spectrum of each spiked sample was collected three times (n=3) and the accuracy was expressed as the mean percentage recovery of three replicates for each spiked sample.

Quantification of AGA based on FTIR method

After validation process, quantification of selected AGAs in theirformulations (ophthalmic preparations and injection) was carried out after evaporating the liquid sample under thermostatically controlled oven and the dried powder was diluted with pure KBr as per calibration curve and the content was determined by directly place the grinded powder on the diamond reader accessory to get the corresponding FTIR spectrum. The quantification was conducted in triplicate and results were expressed as mean ± standard deviation.

3. Results and Discussion

Gentamicin

Method development

During the development of ATR-FTIR method, the spectra of gentamicin standard were collected initially as a step in the qualification and quantification process.Full spectrum of pure gentamicin sulphatewas scanned from 4000-650 cm^{-1} and overlaidwith external spectrum obtained from Spectrabase (CAS Registry Number of 1405-41-0) (19). The overlaid spectrum was displayed in Figure 2. A set of 9 gentamicin working standards with wide range from 0.25% to 15% w/w was used to generate a calibration curve. Full spectra of pure standard of concentrations ranging 0.25-15% w/w and its calibration curve were shown in Figure 3 and Figure 4.

The FTIR spectra showed both N-H group, including secondary anime (3450-3350 cm^{-1}) and primary amine (1650-1550cm^{-1}), C-H stretch group ranging 3040-2940 cm^{-1}, methyl group ranging 1540-1490 cm-1with bending vibration, tertiary alcohol group ranging 1150-1085 cm^{-1}, di-alkyl ether group ranging 1070-1030cm^{-1}, and out-of-plane amine bend at 900-850cm^{-1}. The

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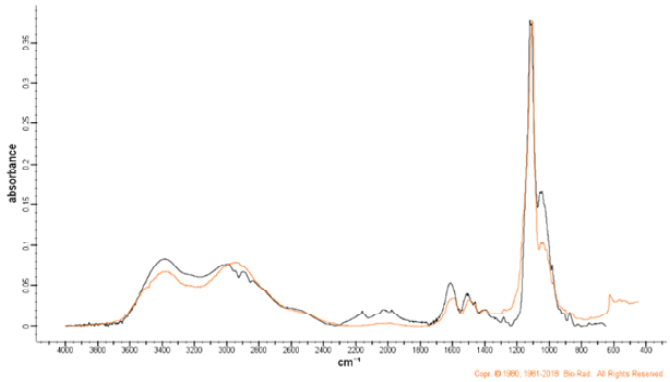

Figure 2. Spectrum of gentamicin standard overlaid with external standard spectrum in full infrared region (4000-650 cm^{-1}).

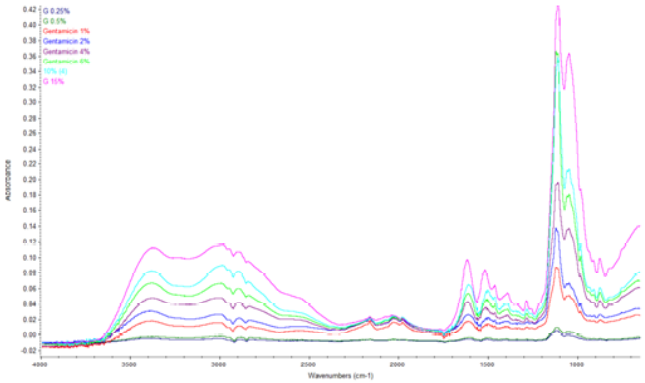

Figure 3. Full spectrum of gentamicin standard with increasing concentration in full infrared region (4000-650cm^{-1}).

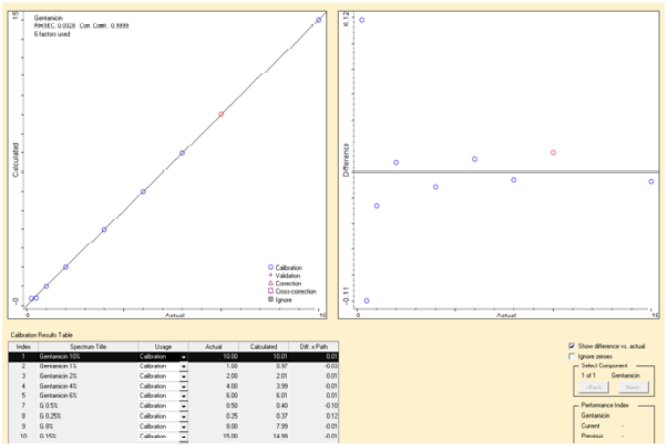

Figure 4. The calibration curve of gentamicin standard full spectrum ranging 0.25% to 15% w/w with correlation coefficient r^2 of 0.9999.

interpretation of major peaks and their correspondence to the chemical groups in gentamicin chemical structure were shown in Table 1.

Band 1 (secondary amine) and band 4 (methyl group)were the unique functional groups to ease differentiation process of gentamicin sulphate from other aminoglycosides. Band 1 is a secondary amine (R'R"CH-NH-CH3) stretching vibration and band 4is amethyl group (CH3C-R) with bending vibration. Both peaks were identified with strong intensity and met the selection criteria.The absorbance and calibration curve of standard 1% w/w at 3381cm^{-1}.Since band 1 has higher correlation

Table 1: List of IR Band Assignments of predominant chemical groups present in Gentamicin Sulphate

Bonds		Wavenumber (cm^{-1})		Possible Functional Group	Vibration	r^2value
Name	Intensity*	Theoretical	Experimental			
N-H	M	3350-3310	3450-3350	Secondary amine	stretch	1.0000
C-H	M-S	3000-2850	3040-2940	Alkane	stretch	0.9938
N-H	W-M	1650-1580	1650-1550	Amine	bend	0.9998
C-H	M-S	1470-1450	1540-1490	Methyl group	bend	0.9975
C-O-H	S	1205-1125	1150-1085	Tertiary alcohol	stretch	0.9994
C-O-C	S	1150-1085	1070-1030	Di-alkyl ether	stretch	0.9690
N-H	-	800	900-850	Amine	out of plane bending	0.9997

*Intensity abbreviations: S- Strong; M- Medium; W- Weak;

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coefficient value, secondary amine was selected as main functional group.

FTIR Method Validation

Average height locations of peaks were used for the generation of calibration curve by scanning major peaks from 4000cm^{-1} to 650cm^{-1} to find specific bands that could provide highest correlation coefficient (r^2) with value greater than 0.995. The correlation coefficient (r^2) values for calibration curve in full infrared region corresponding to each band was listed in Table 2 below.

Table 2: R^2 values for calibration curve at each band based on PLS method for full ATR-FTIR spectrum (4000-650cm^{-1}) of gentamicin standard.

Band	Wavenumber (cm^{-1})	Correlation Coefficient (r^2)	Root Mean Squared Error Calibration (RMSEC)
1	3450-3350	1.0000	0.0254
2	3040-2940	0.9938	0.3840
3	1650-1550	0.9998	0.0021
4	1540-1490	0.9975	0.2420
5	1150-1085	0.9980	0.2180
6	1070-1030	0.9690	0.8530
7	900-850	0.9997	0.0861

Table 3: Linearity, LOD and LOQ values of gentamicin standards at band 1 single spectrum

Spectrum	Linear range (%w/w)	Correlation coefficient (r^2)	RMSEC	LOD (%w/w)	LOQ (%w/w)
Band 1	0.25-15	1.0000	0.0254	0.2006	0.6080

Table 4: Intra-day precision of gentamicin standard using simple Beer's Law at band 1and band 4 single spectra

Analyte Concentration (%)	Selected band Wavelength (cm^{-1})	Intra-day precision Reading (n=3)	Mean ± SD	Intra-day RSD (%)
0.5	Band 1	1: 0.54 2: 0.55 3: 0.53	0.54 ± 0.0100	1.852
	Band 4	1: 0.52 2: 0.50 3: 0.53	0.52 ± 0.0153	2.957
4.0	Band 1	1: 4.02 2: 3.92 3: 4.05	3.99 ± 0.0681	1.703
	Band 4	1: 4.02 2: 3.90 3: 4.08	4.00 ± 0.0916	2.291
10.0	Band 1	1: 9.84 2: 10.06 3: 10.09	9.99 ± 0.1365	1.365
	Band 4	1: 10.04 2: 9.98 3: 9.95	9.99± 0.0458	0.459

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The calibration curve of band 1 was indicated with correlation coefficient of 1.000 with good linearity. The method was considered sensitive with LOD and LOQ values of 0.2006% w/w and 0.6080% w/w respectively. The linearity and sensitivity results of this method for gentamicin standards at band 1 (3450-3350cm^{-1}) single spectrum was shown in Table 3. Linearity was indicated by range value (%w/w) with its r^2 and RMSEC values, while sensitivity of the test was shown with LOD and LOQ values. The %RSD values of intra-day precision were around 2%, and inter-day precision was achieved less than 4% with low deviation among triplicates. The calculated %RSD values for intra-day and inter-day precision were stated in Table 4 and Table 5.

Quantification of gentamicin in ophthalmic preparations

The validated proposed ATR-FTIR method was used for quantification of gentamicin eye drop formulation. The gentamicin concentration was calculated using regression equation from calibration curve using TQ Analyst software. The mean of labelled amount was found to be 104± 2.9143mg with (%RSD 2.799).

Commercial gentamicin ophthalmic preparation (Beagenta eye/ear drops(ED) 0.3% w/v, 5mL capacity)was procured from local pharmacy and dried in Binder RE53 Gravity Convection Ovenr.at 50oCto evaporate liquid to obtain a white colored powder. Drying conditions were optimized by drying three samples of the same batch at 25^{o}C, 40^{o}C and 50^{o}C temperature and dried in desiccator as well. Gentamicin is well noted for being a heat stable antibiotic, retaining its activity even after autoclaving (20-22). The full spectrum of dried gentamicin 0.3% w/v ophthalmic formulation sample with excipient benzalkonium chloride 0.1% w/v.

Peak of tertiary alcohol group (band 5) is shifted towards1191cm^{-1}, however secondary amine (band 1) is

Table 5: Inter-day precision of gentamicin standard using simple Beer's Law at band 1and band 4 single spectra

Analyte	Selected band	Inter-day precision		
Concentration (%)	**Wavelength (cm^{-1})**	**Reading (n=3)**	**Mean ± SD**	**Intra-day RSD (%)**
0.5	Band 1	1: 0.46 2: 0.48 3: 0.45	0.46 ± 0.0152	3.297
	Band 4	1: 0.53 2: 0.54 3: 0.57	0.547 ± 0.0208	3.808
4.0	Band 1	1: 3.84 2: 3.98 3: 4.15	3.990 ± 0.1552	3.891
	Band 4	1: 3.81 2: 4.12 3: 4.03	3.987 ± 0.1595	4.000
10.0	Band 1	1: 9.71 2: 9.75 3: 10.33	9.930 ± 0.3470	3.494
	Band 4	1: 9.80 2: 9.85 3:10.40	10.017 ± 0.3329	3.324

Table 6: Result of quantification of three gentamicin dried ED formulation in powder form

Bottle	Empty beaker weight (mg)	Beaker + Sample weight (mg)	Sample weight (mg)
1	33259.2	33359.8	100.8
2	32710.8	32817.0	106.2
3	26057.0	26162.4	105.4
	Mean ± SD of sample weight (mg)		104.1 ± 2.9143
RSD (%)			2.7987

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totally masked by broad OH spectrumranging wavenumber 3400-3000cm^{-1}. Since secondary amine could not be detected in this case, tertiary alcohol group will be the focus of the study to quantify gentamicin in selected preparation.Another three bottles of same batch were dried at the same time optimized conditions. Average weight of powder was obtained and tabulated in Table 6.

Samples with 5 different concentrations (0.25%, 0.5%, 1%, 2% and 4%) were prepared from dried sample powder. Sample linearity curve was plotted in Figure 5. Sample spectrum was shown in (Figure 5)

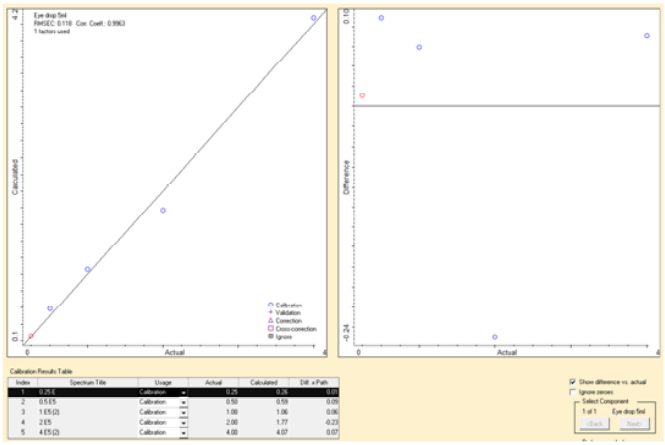

Figure 5. 0.3% w/v Gentamicin ophthalmic solution sample linearity curve with concentration ranging 0.25% - 4%.

Recovery studies

The accuracy was evaluated by calculating the percentage recovery of gentamicin sample. The driedgentamicin sample powder (1% w/w) was accurately weighed and spiked with different concentrations (0.4%, 1%, and 5% w/w of gentamicin) of pure standards using KBr as diluent andtopped up to 100mg. The FTIR spectrum of each spiked sample was collected three times and the accuracy was expressed as the mean percentage recovery of three replicates for each spiked sample. The calculation was carried out by the given equation below(23,24).

$$R\ (\%) = [(C-B)/A] \times 100\%$$

where,

R = Percentage recovered,

A = Amount of known standard (exogenous addition),

B = Known sample concentration before addition, and

C = Total concentration measured after addition.

The statistics of recovery test were revealed with high recovery performance (98.75 -103.2%)with mean recovery percentage of 100.727% ± 2.3597 as shown in in Table 7. The sampling mean was followed a normal distribution. In this case, the standard error of the mean (SEM) was calculated using the equation below .

$$s_x = \frac{S}{\sqrt{N}} = 1.3624$$

The mean recovery was significant with margin of error(100 ±2.65% at 95% confidence interval). The recovery results were clearly indicated that there was no significant interferenceobserved from any excipients present in the matrix and hence this method was proven to be feasible without any solvent extraction. According to USP, gentamicin sulphate ophthalmic preparation contains equivalent of not less than 90% and not more than 135% of the labelled amount of gentamicin(5).

Method development

The primary step in qualification process of ATR-FTIR method is to obtain the spectra The FTIR spectra of tobramycin standard.at full infrared region (4000-650 cm^{-1}) was shown in Figure 6(a).The IR spectra was showed peaks corresponding to the functional groups in the chemical structure that could be used for qualitative and quantitative analysis of tobramycin. The external standard spectrum of Tobramycin with KBr discwas obtained from SDBS with CAS Registry Number of 32986-56-4 is shown in Figure 6(b)(25).

Different concentrations of tobramycin standards ranging from 0.25% to 15% were prepared and FTIR

Table 7. Recovery test of gentamicin from samples after exogenous addition of known standards concentration.

Amount of known standard in % (A)	Known sample concentration before addition in % (B)	Total concentration measured (C)	%RSD	Recovery Efficiency (R)
0.4	1	1.395 ± 0.0458	3.273	98.75%
1	1	2.032 ± 0.0436	2.179	103.20%
5	1	6.024 ± 0.1015	1.691	100.48%

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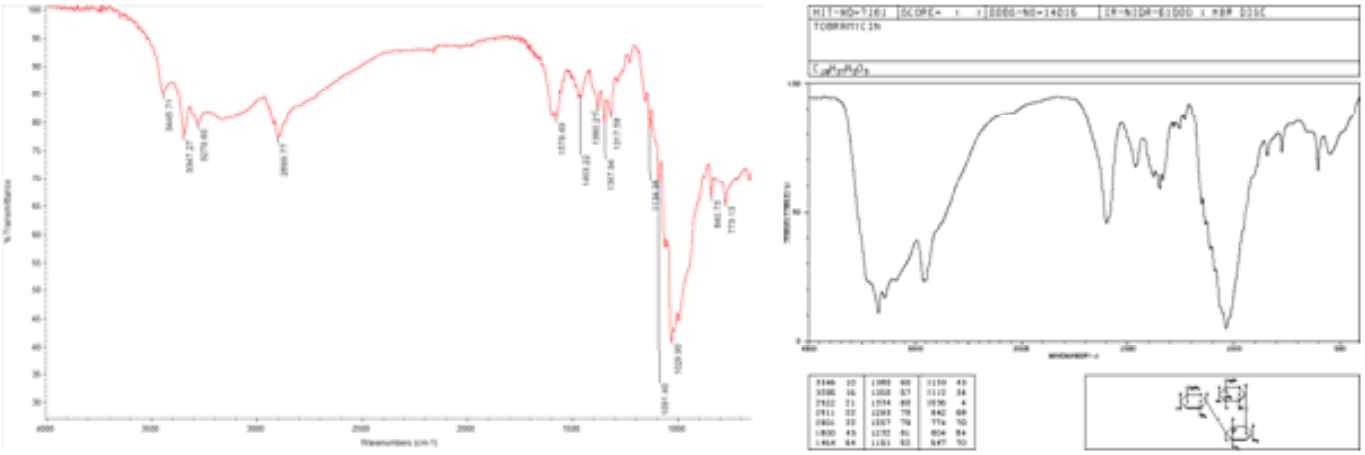

(a) **(b)**

Figure 6. (a) Pure tobramycin spectrum scanned at full infrared region (4000-650 cm^{-1}) with ATR-FTIR instrument; (b) External standard spectrum of tobramycin with KBr disk from AIST SDBS with list of displayed peak wavenumber.

graded KBr was used as background spectrum. Figure 7 presents the tobramycin standard spectra with calibration curve in full infrared region. Full spectrum of tobramycin standard showed excellent calibration curve with correlation coefficient r2 value of 0.9999, which is shown in Figure 8.

The major two primary amine stretching at wavenumber 3470-3420cm^{-1} and 3370-3320cm^{-1}, bending at 1620-1550cm^{-1}, alkane stretching at 2915-2875cm^{-1}, methylCH3 -CHbending at a range of 1400-1370cm^{-1},substituted alkane group at 845-835cm^{-1} and 790-755cm^{-1}. As for O-H functional group,alcohol stretch group is ranged within wavenumber 3305-3255cm^{-1}, in-plane OH bending is found at 1360-1340 cm^{-1}, while primary OHgroup was noticed at 1050-1000cm^{-1}. Ether group is also found at 1145-1125cm^{-1}. The interpretation of major peaks and their correspondence to the chemical groups in tobramycin chemical structure are shown in Table 8.

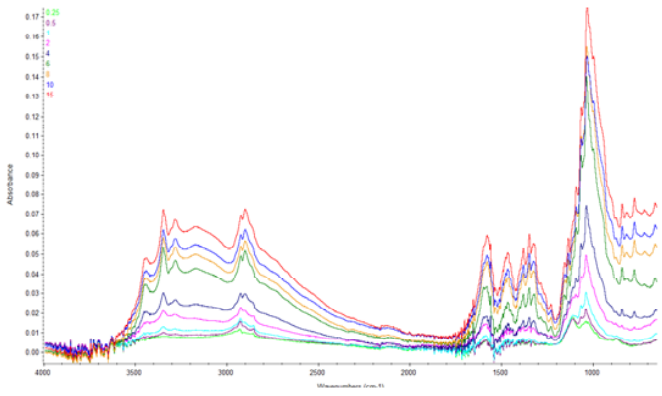

Figure 7. Spectra of tobramycin standards with increasing concentration in full infrared region (4000-650cm^{-1}).

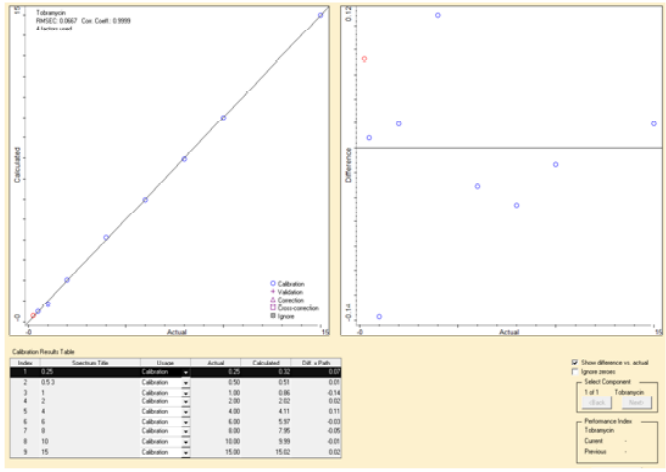

Figure 8. The calibration curve of tobramycin standard full spectrum ranging 0.25% to 15% with r^2 value of 0.9999.

Peaks corresponding to hydroxide, alkane and ether functional groups were not considered for quantification purpose. In this study, band 2 (3375-3325cm^{-1}) single spectrum with r^2 value of 0.9998 and band 10 (1050-1000cm^{-1}) single spectrum with r^2 value of 0.9992 are selected for the qualitative analysis of since they are the unique functional group of tobramycin as compared to gentamicin. The peak height location of band 2 is displayed at 3347.3cm^{-1}.

Method validation

Average height locations of peaks were used for the generation of calibration curve by scanning major peaks from 4000cm^{-1} to 650cm^{-1} to find specific bands that could provide highest correlation coefficient (r^2) with value more than 0.995. Table 9 showed the r^2 values of calibration curve corresponding to each band.

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Table 8: List of major IR band assignments of predominant chemical groups present in tobramycin.

Bonds		Wavenumber (cm^{-1})		Possible Functional Group	Vibration	rvalue
Name	Intensity*	Theoretical	Experimental			
N-H	M	~3500	3470-3420	Primary amine	Stretch	0.9803
N-H	M	3400-3300	3370-3320	Aliphatic primary amine	Stretch	0.9999
O-H	M, broad	3400-3300	3305-3255	Alcohol	Stretch	1.0000
C-H	M	3000-2840	2915-2875	Alkane	Stretch	0.9978
N-H	M	1650-1580	1620-1550	Primary amine	Bend	0.9939
C-H	W	1470-1450	1475-1450	Alkane	Bend	0.9940
CH_3 .CH	M	1470-1450	1400-1370	Methyl group	Bend	0.9929
O-H	M	1390-1310	1360-1340	Alcohol	In-plane bending	0.9986
R-O-R	M	1150-1085	1145-1125	Aliphatic ether	Stretch	0.9992
CH_2OH	M-S	1085-1050	1050-1000	Primary alcohol	Stretch	0.9996
C-H	M	810±20	845-835	1,4-disubstituted alkane	Bend	0.9919
C-H	M	780±20	790-755	1,2,3-trisubstituted alkane	Bend	0.9940

*Intensity abbreviations: S- Strong; M- Medium; W- Weak;

Table 9: Correlation coefficient values for calibration curve at each band based on PLS method.

Band	Wavenumber (cm^{-1})	Correction Coefficient (r^2)	Root Mean Squared Error Calibration (RMSEC)
1	3470-3420	0.9610	0.939
2	3375-3325	0.9998	0.063
3	3305-3255	1.0000	0.016
4	2915-2875	0.9956	0.315
5	1620-1550	0.9878	0.526
6	1475-1450	0.9880	0.521
7	1400-1370	0.9859	0.564
8	1360-1340	0.9972	0.247
9	1145-1125	0.9984	0.195
10	1050-1000	0.9992	0.448
11	845-835	0.9839	0.606
12	790-750	0.9912	0.140

The calibration curve of Band 2 was indicated with good linearity with r^2 value of 0.9998. The method was considered sensitive with calculated LOD and LOQ values of 0.2296% w/w and 0.7654% w/w respectively. Table 10 shows the linearity and sensitivity results of this method for tobramycin standards at band 2(3375 - 3325cm^{-1}) single spectrum. The %RSD of intra-day readings were around 2, while %RSD were lower than 4 among triplicate inter-day readings, with data shown in Table 11 and 12.

Quantification of tobramycin in drug formulation

The quantification of tobramycin eye drop was tested using the validated ATR-FTIR method. The tobramycin concentration was calculated using regression equation from calibration curve using TQ Analyst software. The mean of labelled amount was found to be 84.57mg ± 2.658, with %RSD of 3.15.

Tobrexophthalmic solution 0.3% w/v formulation in 5mL bottle was procured from local pharmacy and dried

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Table 10: Linearity, LOD and LOQ values of tobramycin standards at single spectrum

Spectrum	Linear range (%w/w)	Correlation coefficient (r^2)	RMSEC	LOD (% w/w)	LOQ (%w/w)
Band 2	0.25-15	0.9998	0.0626	0.2296	0.7654

Table 11: Intra-day precision of tobramycin standard using simple Beer's Law at single spectra

Analyte	**Selected band**	**Intra-day precision**		
Concentration (%)	**Wavelength (cm^{-1})**	**Reading (n=3)**	**Mean ± SD**	**Intra-day RSD (%)**
0.5	Band 2	1: 0.51 2: 0.49 3: 0.51	0.503 ± 0.0115	2.294
	Band 10	1: 0.52 2: 0.49 3: 0.50	0.503 ± 0.0153	3.035
4.0	Band 2	1: 3.94 2: 4.04 3: 4.02	4.000 ± 0.0529	1.323
	Band 10	1: 4.01 2: 4.01 3: 3.97	3.997 ± 0.0231	0.578
10.0	Band 2	1: 10.01 2: 9.99 3: 9.99	9.997 ± 0.0115	0.116
	Band 10	1: 10.03 2: 10.10 3: 9.87	10.000 ± 0.1179	1.179

Table 12: Inter-day precision of tobramycin standard using simple Beer's Law at single spectra

Analyte	**Selected band**	**Inter-day precision**		
Concentration (%)	**Wavelength (cm^{-1})**	**Reading (n=3)**	**Mean ± SD**	**Inter-day RSD (%)**
0.5	Band 2	1: 0.49 2: 0.50 3: 0.51	0.500 ± 0.0100	2.000
	Band 10	1: 0.48 2: 0.52 3: 0.51	0.503 ± 0.0208	4.136
4.0	Band 2	1: 4.10 2: 4.11 3: 3.87	4.027 ± 0.1357	3.372
	Band 10	1: 3.96 2: 4.05 3: 3.99	4.000 ± 0.0458	1.146
10.0	Band 2	1: 10.05 2: 10.05 3: 9.89	9.997 ± 0.0924	0.924
	Band 10	1: 9.81 2: 10.10 3: 10.09	10.000 ± 0.1646	1.646

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Table 13: Average weight of three dried ophthalmic formulation sample

Bottle	Empty beaker weight (mg)	Beaker + Sample weight (mg)	Sample weight (mg)
1	26047.8	26134.0	86.2
2	32728.2	32809.7	81.5
3	33003.4	33089.4	86.0
	Mean ± SD of sample weight (mg)		84.57 ± 2.658
RSD (%)			3.15

at similar condition as gentamicin to obtain white colored powder. As mentioned in several reports, tobramycinretains stability in high temperature, retaining its activity even after autoclaving (20,26). The full spectrum of tobramycin 0.3% w/v ophthalmic solution with excipient benzalkonium chloride 0.1% w/v.

Peak of primary amine (band 2) is being masked by OH group at 3203.22cm-1, while band 10 representing primary OH group is still visible at wavenumber 1025.07cm-1. Since primary amine could not be detected in this case, band 10 will be the focus of the study to quantify tobramycin in selected formulation. Another three bottles of same batch (Batch no: 18K25AG) were dried at the same time in same condition. Average weight of powder is obtained and tabulated in Table 13.

Sample with concentrations 0.25%, 0.5%, 1%, 2% and 4%were prepared to plot linearity curve. Sample linearity curve and spectrum could be seenrespectively.

Recovery studies

The statistics of recovery studieswere revealed high recovery performance (99.29 -103.00 %), with mean recovery percentage of 101.04% ± 1.864 as shown in Table 14.Since sampling mean was followed a normal distribution, the standard error of the mean (SEM) was 1.076. The mean was significant (with margin of error of ±2.09%) at 95% confidence interval. The recovery results revealed that there is no significant interference from any excipients present in the matrix and hence proven that this method is feasible without any solvent extraction.

According to USP 39, tobramycin ophthalmic solution contains equivalent of not less than 90% and not more than 120% of the labelled amount of tobramycin.(5)These results clearly prove the validity of proposed direct method using ATR-FTIR with PLS method for quantitative analysis of tobramycin from its ophthalmic formulation.

Kanamycin

Method Development

Full spectrum of kanamycin standard was scanned from 4000 to 650cm-1 wavenumber range and the spectrum was overlaid with an external spectrum obtained from Spectrabase (CAS Registry Number of 25389-94-0), as shown in Figure 9. The calibration curve was obtained and shown in Figure 10with good calibration coefficient greater than 0.995.

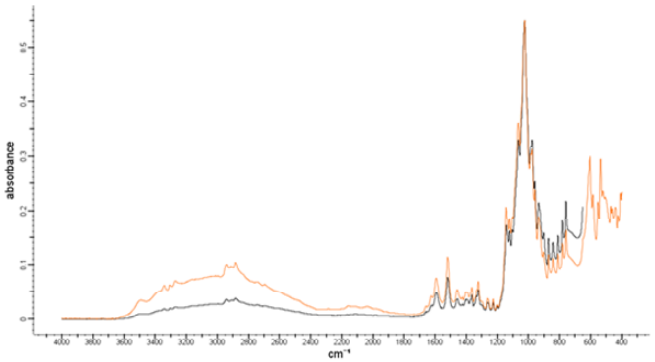

Figure 9. Spectrum of kanamycin standard overlaid with external standard spectrum in full infrared region.

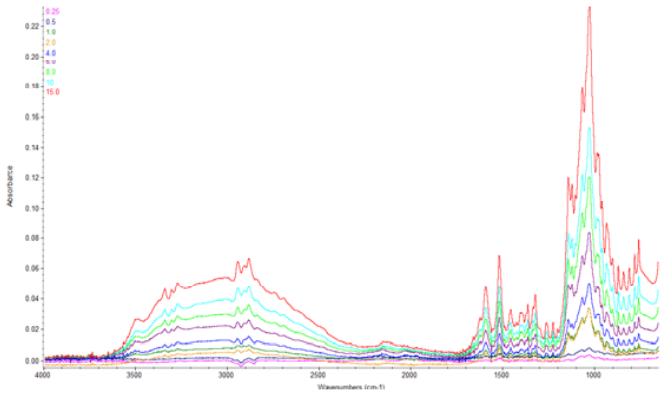

Figure 10. Full spectrum of kanamycin standard with increasing concentration in full infrared region (4000-650cm^{-1}).

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Table 14. Recovery studies of tobramycin from samples after exogenous addition of known standards concentration.

Amount of known standard in % (A)	Known sample concentration before addition in % (B)	Total concentration measured (C)	RSD (%)	Recovery Efficiency (R)
0.4	1	1.390 ± 0.0400	2.878	99.29%
1	1	2.060 ± 0.0265	1.284	103.00%
5	1	6.050 ± 0.0265	0.437	100.83%

Table 15: List of major IR band assignments of predominant chemical groups present in kanamycin sulphate.

Bonds		Wavenumber (cm^{-1})		Possible Functional Group	Vibration	rvalue
Name	Intensity*	Theoretical	Experimental			
N-H	M	~3500	3520-3460	Primary amine	Stretch	0.9880
N-H	M	3400-3300	3355-3325	Aliphatic primary amine	Stretch	0.9956
C-H	M	3000-2840	2895-2870	Alkane	Stretch	0.9958
N-H	S	1650-1580	1610-1575	Primary Amine	Bend	0.9997
	S		1540-1500			0.9989
C-H	W	1450	1465-1445	Methyl group	Bend	0.9915
O-H	W	1390-1310	1365-1355	Alcohol	In-plane bending	0.9992
C-N	W	1250-1020	1230-1215	Amine	Stretch	0.9981
R-O-R	M	1150-1085	1145-1135	Aliphatic ether	Stretch	0.9913
O-H	S	1124-1087	1125-1115	Secondary alcohol	Stretch	0.9734
C-O	S	1085-1050	1070-1055	Primary alcohol	Stretch	0.9998
S=O	S	1070-1030	1040-1010	Sulphoxide	Stretch	0.9994
C-H	M	880±20	875-860	1,2,4-trisubstituted or 1,3-disubstituted alkane	Bend	0.9966
C-H	M	810±20	845-835	1,4-disubstituted alkane	Bend	0.9895
			815-800			0.9880
C-H	M	780±20	785-775	1,2,3-trisubstituted alkane	Bend	0.9865
C-H	M	750±20	765-755	Monosubstituted alkane	Bend	1.0000

*Intensity abbreviations: S- Strong; M- Medium; W- Weak;

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The spectra displayed several important functional groups in kanamycin structure, including primary amine stretching group at wavenumber 3520-3460 cm^{-1}, aliphatic primary amine ranging 3355-3325 cm^{-1}, primary amine bending groups (1610-1575 cm^{-1}, 1540-1500cm^{-1}), methyl group at 1465-1445cm^{-1}, and several substituted alkanes groups (875-860cm^{-1}, 845-835cm^{-1}, 785-775cm^{-1}, 765-755cm^{-1} etc). Due to similarity in structure with tobramycin, primary alcohol group could be found in kanamycin spectra, ranging at wavenumber 1070-1055cm^{-1}, and aliphatic ether peak of kanamycin could be found at 1145-1135cm^{-1} wavenumber as well. Since kanamycin has more secondary alcohol group and lesser primary amine group than tobramycin, the spectra of both AGAs showed some significant difference especially in wavenumber ranging 3500-3300cm^{-1} and 1650-1580cm^{-1} which usually depict amine functional group. The difference in spectra could be noticed in both spectra.

Tobramycin peak corresponding to hydroxide, alkane and ether functional groups were not considered for quantification purpose , but certain bands were shown in Table 15, band 4 (1610-1575cm^{-1}) single spectrum with r^2 value of 0.9994 and band 5 (1540-1500cm^{-1}) single spectrum with r^2 value of 0.9978 were selected for analysis. The selected peaks were sharp and non-overlapping unlike tobramycin.

Method validation

Average height locations of peaks were used for the generation of calibration curve by scanning major peaks from 4000cm^{-1} to 650cm^{-1} to find specific bands that could provide highest correlation coefficient (r2) with value more than 0.995 and the r^2 values of calibration curve corresponding to each functional group in kanamycin as shown in Table 16.

The calibration curve of Band 4 was indicated with good linearity with r^2 value of 0.9994. The method was considered sensitive with calculated LOD and LOQ values of 0.2393% w/w and 0.7977% w/w respectively. The linearity and sensitivity results for kanamycin standards at band 4 (1610-1575cm^{-1}) single spectrum were shown in Table 17. The %RSD among triplicate, the intra-day and inter-day readings were less than 2.00 and 4.00 as shown in Table 18 and 19.

Table 16: Correlation coefficient values for calibration curve at each band based on PLS method

Band	Wavenumber (cm^{-1})	Correction Coefficient (r^2)	RMSEC
1	3520-3460	0.9761	0.733
2	3355-3325	0.9912	0.446
3	2895-2870	0.9914	0.434
4	1610-1575	0.9994	0.118
5	1540-1500	0.9978	0.222
6	1465-1445	0.9831	0.618
7	1365-1355	0.9984	0.193
8	1230-1215	0.9962	0.296
9	1145-1135	0.9827	0.627
10	1125-1115	0.9475	1.090
11	1070-1055	0.9996	0.092
12	1040-1010	0.9988	0.160
13	875-860	0.9932	0.389
14	845-835	0.9791	0.688
15	815-800	0.9761	0.734
16	785-775	0.9732	0.779
17	765-755	1.0000	0.033

Table 17: Linearity, LOD and LOQ values of kanamycin standards at single spectrum

Spectrum	Linear range (%w/w)	Correlation coefficient (r^2)	RMSEC	LOD (% w/w)	LOQ (%w/w)
Band 4	0.25-15	0.9994	0.118	0.2393	0.7977

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Table 18: Intra-day precision of kanamycin standard using simple Beer's Law at single spectra

Analyte	Selected band	Intra-day precision		
Concentration (%)	**Wavelength (cm^{-1})**	**Reading (n=3)**	**Mean ± SD**	**Intra-day RSD (%)**
0.5	Band 4	1: 0.51 2: 0.49 3: 0.50	0.50 ± 0.010	2.000
	Band 5	1: 0.50 2: 0.48 3: 0.52	0.49 ± 0.020	4.000
4.0	Band 4	1: 4.00 2: 4.01 3: 3.98	3.99 ± 0.015	0.382
	Band 5	1: 4.00 2: 4.01 3: 3.99	4.00 ± 0.010	0.250
10.0	Band 4	1: 9.81 2: 10.03 3: 10.16	10.0 ± 0.177	1.769
	Band 5	1: 9.76 2: 10.01 3: 10.13	9.97 ± 0.189	1.894

Table 19: Inter-day precision of kanamycin standard using simple Beer's Law at single spectra

Analyte	Selected band	Inter-day precision		
Concentration (%)	**Wavelength (cm^{-1})**	**Reading (n=3)**	**Mean ± SD**	**Intra-day RSD (%)**
0.5	Band 4	1: 0.51 2: 0.52 3: 0.54	0.52 ± 0.015	2.919
	Band 5	1: 0.53 2: 0.51 3: 0.55	0.53 ± 0.020	3.774
4.0	Band 4	1: 4.03 2: 4.10 3: 3.87	4.0 ± 0.118	2.947
	Band 5	1: 4.00 2: 4.12 3: 3.88	4.0 ± 0.120	3.000
10.0	Band 4	1: 9.71 2: 10.03 3: 10.26	10.0 ± 0.276	2.762
	Band 5	1: 9.68 2: 10.03 3: 10.29	10.0 ± 0.306	3.061

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Quantification of kanamycin injections

Similar drying method was used on kanamycin formulation, however due to high concentration as compared to gentamicin and tobramycin, gel state was formed. The drying was done with stirring at every one-hour point. Kanamycin white crystalline powder was grinded in big mortar and pestle to ensure uniform mixing with KBr powder.

Full spectrum of kanamycin IM injection sample was shown as a peak of primary amine bending group (band 4) is visible within wavenumber 1650-1600cm^{-1}, which was the focus for the quantification study in selected formulation. Another three vials of kanamycin IM injection were dried at the same time under the same condition. Average weight of powder was obtained and tabulated in Table 20. The mean of labelled amount was found to be 390.63mg ± 5.472, with %RSD of 1.40.

Sample with concentrations from 0.25% to 15% were prepared to plot linearity curve. Sample calibration curve was shown with good linearity.

Recovery studies

The statistics of recovery studieswere revealed high recovery performance (99.92 -101.75 %), with mean recovery percentage of 100.67% ± 0.960 as shown in Table 21. The sampling mean was followed a normal distribution, the standard error of the mean (SEM) wasestimated to be 0.554. The mean was significant (with margin of error of ±1.08%) at 95% confidence interval. The recovery results were revealed that there was no significant interference from any excipients present in the matrix. According to USP, Kanamycininjection must meet the approval criteria of an amount of Kanamycin Sulfate equivalent to not less than 90.0 percent and not more than 115.0 percent of the labeled amount of kanamycin.(5).The results were proved the validity of proposed ATR-FTIR spectroscopy method for quantitative analysis of kanamycin injection.

LC-MS Reference Method

Gentamicin

Gentamicin is usually found in mixture of five components (C1, C1a, C2, C2a, and C2b). ESI mass spectrum was recorded from Gentamicin standard and it was identified as mass spectral peaks that represented the protonated species of all Gentamicin subtypes (m/z 478, 450, and 464), The molecule with the greatest m/z value indicates the parent ion.

Good linearity of Gentamicin standard was shown withgood correlation coefficient (r2 0.9986). For recovery studies, the percent purity was calculated according to the formula below.

$$\% \text{ Purity} = \frac{Cun}{Cstd} \times 100$$

Where C_{un} is concentration of sample,
C_{std} is concentration of standard.

The recovered concentration was 1.457µg/mL, therefore the calculated recovery percentage was 112%.

Table 20: Average weight of each of the three dried injection vial sample

Bottle	Empty beaker weight (mg)	Beaker + Sample weight (mg)	Sample weight (mg)
1	27463.4	27859.2	395.8
2	32935.1	33320.0	384.9
3	32618.8	33010.0	391.2
	Mean ± SD of sample weight (mg)		390.63 ± 5.472
RSD (%)			1.40

Table 21. Recovery test of kanamycin from samples after exogenous addition of known standards concentration.

Amount of known standard in % (A)	**Known sample concentration before addition in % (B)**	**Total concentration measured (C)**	**RSD (%)**	**Recovery Efficiency (R)**
0.4	1	1.405 ± 0.0134	0.956	100.33%
1	1	2.035 ± 0.0567	2.785	101.75%
5	1	5.995 ± 0.0353	0.589	99.92%

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In the reported reference method by Freneiletc, the sensitivity was established, and the results was found to be 1ng/mL for LOD. The percent RSD of intra-day (n=5) and inter-day precision (n=15) were 6.1 and 8 respectively (27).

Tobramycin

ESI operated in positive ion mode was used to study the fragmentation behavior of tobramycin and its known related substances. Tobramycin was yielded a [M+H]+ base peak at m/z 468. Another major spectral peak was collected at m/z 288, which was a major product ion in the fragmentation pathway. It was revealed from the literature about the fragmentation pathway of tobramycin to its major product ion with their m/z value. The LC-MS spectrum of reference method was optimized and established with the optimized conditions.

The good linearity was established with LC-MS reference method with prevailing conditions with good correlation coefficient (r2 0.9931). The concentration was recovered from sample as 1.0411ug/mL, recovery results were found to be 92%. The sensitivity was established in the reported reference study, and the results was found to be 5ng/mL for LOD. The percent RSD of intra-day (n=5) and inter-day precision (n=15) were reported as 5.2 and 7.8 in Freneil's study (27,28).

Statistical analysis

Gentamicin and Tobramycin ophthalmic preparations using the proposed ATR-FTIR method was compared with recovery efficiency obtained from LCMS reference method using independent T-test. Since p value is greater than 0.05, there was no significant difference in recovery efficiency of Gentamicin between proposed method and reference method. Similar results were obtained for Tobramycin as well. The SPSS group statistics table and independent sample test results were conducted and established.

In one-way ANOVA, the ophthalmic preparations recovery studies using ATR-FTIR method among three AGAs wereproved to be non-significant ($p>0.05$). Furthermore, Post-hoc test was presented non-significant difference between the recovery comparison of Gentamicin with Tobramycin; Tobramycin with Kanamycin; and Kanamycin with Gentamicin. One-way ANOVA, descriptive table and Post-hoc test is displayed excellent results.

4. Conclusion

ATR-FTIR instrument has the potential of carrying rapid analysis of different AGAs in commercial preparations. The relationship between IR spectra of the compound and its chemical structure were fully exploited before establishing analytical method validation parameters for detection and quantification of the selected AGAs separately. ATR-FTIR spectroscopy with PLS algorithm for quick quality control analysis as a direct method which does not require any complex derivatization, chemical modification or solvent intervention for the sample preparation except drying. Validation results of the present study were shown precise, accurate and reproducible. These methods were faster, simple and eco-friendly for sensitive detection and accurate measurement of purity of active ingredient from its pharmaceutical preparations.

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Assessment of Knowledge, Attitude and Practice of Malaysian Women Towards Osteoporosis

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Abstract

Due to the increasing proportions of aging populations in the Asian region, osteoporosis has become more prevalent and increases the health care expenditure in this region. The majority of osteoporotic fractures occur in postmenopausal women. It is important to identify women at the highest risk and to prevent further fractures. We aimed to assess knowledge, attitude and practice towards osteoporosis among Malaysian women in Klang Valley.A cross-sectional study was conducted in 384 Malaysian women aged above 18 years. A researcher-administered questionnaire was used to collect data. The participants were selected conveniently from obstetrics and gynecology (O&G) or orthopedic clinics from 6 districts of Klang Valley. Data analysis was done by SPSS version 22, using ANOVA, t-test, Chi-square test and Pearson correlation. The findings show participants had a poor score of knowledge towards osteoporosis. There was a significant association between the level of osteoporosis knowledge and education level, employment status and occupation of participants ($P<0.05$). The participants had a moderate attitude towards osteoporosis. Age, race and education level of participants was significantly associated with the attitude towards osteoporosis. Most participants had poor preventive practices against osteoporosis. The level of practice to prevent osteoporosis was significantly associated with races, education level, occupation and monthly income of participants. Both knowledge and attitude towards osteoporosis were correlated with the practices to prevent osteoporosis.The participants had inadequate knowledge, moderate attitude and poor practice towards osteoporosis. This could serve as a stimulant for policymakers to increase the education of osteoporosis among younger women. Furthermore, the practice against osteoporosis among high-income participants was higher than low-income. This indicates that poverty should be addressed in Malaysia.

Key words : Osteoporosis, knowledge, attitude, practice, Malaysian women

1. Introduction

Osteoporosis is a common disease characterized by a systemic impairment of bone mass and microarchitecture that results in increase bone fragility and susceptibility to fracture (1). Due to the increasing proportions of aging populations in the Asian region, osteoporosis has become more prevalent and increases the health care expenditure in this region (2). In Malaysia, 5.3 million of the population was aged 50 and above in 2013 and the number is expected to increase rapidly to 13.9 million in 2050 (3). In 2000, WHO estimated that osteoporosis causes more than 8.9 million fractures worldwide every year (4). There is limited data documented on the prevalence of osteoporosis in Malaysia. However, a study in 2005 reported the prevalence of osteoporosis was 24.1%, predominantly at the hip (5).

The majority of osteoporotic fractures occur in postmenopausal women. Therefore, to manage postmenopausal osteoporosis, it is important to identify women at the highest risk and to prevent further fractures (6). There were a few studies that determine the prevalence (3) and knowledge, attitude and practice (7, 8) of osteoporosis among Malaysians. However, there is limited study regarding osteoporosis among Malaysian women.

Postmenopausal osteoporosis is the main consequence of bone loss. It is caused by estrogen deficiency and therefore affects mainly women (9). The incidence of osteoporosis increases markedly with age. Osteoporotic fractures are increasingly prevalent in women after 55 years of age and in men after 65 years of age (10). Ethnicity and race are also important factors to determine the risk of osteoporosis. A survey on hip fracture incidence

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among Malaysian populations showed that 63% were Chinese(11).

Older people with a history of fractures, family history of osteoporosis and history of rheumatoid arthritis are at a particularly higher risk of osteoporotic fractures (9). Modifiable risk factors such as the history of smoking, alcohol abuse, intake of caffeine and history of glucocorticoid use also increase the incidence of osteoporosis (9, 12).

A study shows that self-efficacy for calcium intake and physical exercise directly affect the corresponding practices among young adults (13). Another study in the US suggests that understanding the barriers to calcium supplements is essential to increase calcium intake and reduce the risk of osteoporosis (14). Exercise is a safe and effective way to prevent bone loss in postmenopausal women. However, most studies showed females had an inadequate physical exercise that could protect them from osteoporosis such as Saudi Arabia (15).

The National Osteoporosis Foundation recommends a total calcium intake of 1200 mg per day and a total vitamin D intake of 800 to 1000 IU per day for people aged over 50 to prevent osteoporosis(16). Increased consumption of vegetables and fruits and low consumption of meat has shown to support bone status (17). Hormone replacement therapy, selective estrogen receptor modulators, bisphosphonates, calcitonin, calcium and vitamin D can be used to treat osteoporosis (18).

An existing study revealed that there was a lack of awareness of the diagnosis of osteoporosis and osteoporotic fracture risk among postmenopausal women in the US(19).The attitude towards osteoporosis is dependent on one's beliefs related to the disease(20). Increasing knowledge, correcting health beliefs and promoting osteoprotective practices are effective measures for building and maintaining strong bone throughout ones' lifespan(21).

In Malaysia, the aging population is alarming and urgent action is required to deal with the projected burden of osteoporosis. However, studies show that the calcium and vitamin D intake remains low in Malaysian women who are at higher risk of getting osteoporosis. Most of the studies emphasized to determine the prevalence of osteoporosis among Malaysian women but so far there is not any study that determined the knowledge, attitude and various methods of practice to prevent osteoporosis among Malaysian women. Therefore, we decided to determine the knowledge and attitude of Malaysian women towards osteoporosis. How Malaysian women practice preventing osteoporosis and the determining factors of this practice also have been concerned in the current study.

2. Materials and Methods

The cross-sectional study was conducted from April to July 2018 and July to August 2019 in Klang Valley, Malaysia. A total of 384 Malaysian women aged 18 years and above were recruited in the study. Malaysian women aged below 18, foreigners and those who refused to take part were excluded from the study. 111 samples were recruited in 2018 while the other 273 samples were obtained in 2019.

A two-stage cluster convenient sampling was used to select the studied population. In the first stage, Klang Valley is clustered into six districts. In the second stage, from each district, 3 or more obstetrics & gynecology (O&G) or orthopedic clinics were selected based on the convenience of accessibility of researchers and patient flow in the clinics. From each district, 64 samples were recruited.

To estimate the sample size, a margin of error of less than 5% and a confidence interval of 95% were used in Raosoft sample size calculator(22). The target population size of 1 million and the response rate of 50% were entered. A sample size of 384 women was needed to be recruited in the study.

Ethical consideration **:** Faculty Research and Scholarly Activities Committee at UCSI University approved the research project in February 2018. Ethical approval was obtained from the Medical Research & Ethics Committee of the Ministry of Health Malaysia. An informed consent letter was signed by all respondents before they answer the questions.

Data collection tool **:** To develop the questionnaire, previous studies carried out on women of the same age group in Sri Lanka (2), Saudi Arabia (15) eight European countries (17) and Canada (23)were utilized. The questionnaire items were modified to meet local needs. The language used in the questionnaire was English and the data was collected by face to face interviews. The interviewer was able to communicate in Mandarin and BahasaMelayu, in addition to English. In case the respondent was not proficient in English, the interviewer assisted the respondent to interpret the questions. The questionnaire included four parts: demographic

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characteristics, knowledge, attitude towards osteoporosis and practice/preventive behaviors against osteoporosis.

***Assessment of knowledge* :** The responses to knowledge items include 'yes', 'no' and 'don't know'. Correct answers were scored as 1, while wrong answers and don't know were scored as 0. The total knowledge score ranges from 0 to 14.

***Assessment of attitude* :** Likert scale items (strongly agree, agree, disagree, strongly disagree) were used for the assessment of attitude regarding osteoporosis. Strongly agree responses were scored as 4, agree as 3, disagree as 2 and strongly disagree as 1. The total attitude score ranges from 8 to 32.

***Assessment of practice* :** The items of practice/prevention behaviors against osteoporosis include frequency of intake of calcium-rich food, frequency of physical activity and frequency of exposure to sun. A five-point scale was used to rate the frequencies of each activity: never scored as 1, seldom scored as 2, occasionally scored as 3, often scored as 4 and always scored as 5. Furthermore, the history of the BMD test and history of calcium supplements were included in the assessment of practice against osteoporosis. If there is a history of these preventive measures, 1 point was given for each. The total practice score ranges from 3 to 17.

***Data analysis* :** SPSS version 22 was used for data analysis. Demographic characteristics and KAP scores were summarized using descriptive statistics and expressed as mean ± standard deviation and percentage. Bloom's cut off scale was used to assess the level of knowledge, attitude and practice towards osteoporosis of participants. The mean KAP total scores of 80-100% were classified as good knowledge, a positive attitude, and good practice, 60-79% as moderate, and <60% indicated a poor level of KAP.

The differences in KAP scores by demographic characteristics were tested using the one-way analysis of variance (ANOVA). T-test was used to compare the means of KAP scores between healthcare-related occupations and non-healthcare related occupations. The P-value of less than 0.05 was considered statistically significant. Chi-square analysis with Fisher's exact test was used to test the relationship between race, marital status and employment status with KAP categories (good, moderate and poor). Pearson's correlation was used to identify the relationship between knowledge and practice as well as attitude and practice towards osteoporosis among participants.

3. Results and Discussion

There was a total of 384 completed questionnaires collected. All the participants were women. Most of the participants (49.2%) in the study aged 30-49. There were 179 Chinese, 46 Indian, 153 Malay and 6 other races women enrolled in the study. Most women in the study (61.7%) were married while the other 37.2% of women were single. There were 45.8% of women university-educated, 31.5% secondary school educated, and 6% with no formal education. More than half of the women were employed while 16.7% were housewives. There were only 26% of women worked in healthcare-related industries while the rest had non-healthcare related jobs. Most women (43.2%) had a monthly income of RM 1000 - RM 3000.

The classifications of knowledge, attitude and practice towards osteoporosis among participants are based on Bloom's cut off point (24) according to the mean scores in each dimension. Table 1 presents the mean score and overall level of classification of KAP among the participants. The overall mean scores ± standard deviation for all participants were 6.52 ± 2.609 for knowledge, 22.72 ± 2.674 for attitude and 10.78 ± 2.963 for practice. The participants had a poor level of knowledge and practice, and a moderate level of attitude towards osteoporosis.

Table 1 : Mean ± SD of knowledge, attitude and practice score towards osteoporosis among participants.

Dimension	Total (384) Mean ± SD (% of the total score)	Level of classification
Knowledge	6.52 ± 2.609 (46.6%)	Poor
Attitude	22.72 ± 2.674 (71%)	Moderate
Practice	10.78 ± 2.963 (63.4%)	Poor

Table 2 presents the result of the association between demographic characteristics and KAP scores using ANOVA and t-test. ANOVA test showed knowledge score was statistically associated with education level and employment status (P<0.05). Attitude score was associated with age, race and education level. While practice score was significantly associated with race, education level and monthly income. T-test showed that women with healthcare-related occupation had significantly higher mean knowledge and practice scores than non-health-care related jobs (both P<0.001).

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Table 2 : The association between demographic variables and KAP scores (n=384), based on ANOVA and t test of significance.

Variables	**Categories**	**N**	**Knowledge Mean ± SD**	**Attitude Mean ± SD**	**Practice Mean ± SD**
Age	18-29	139	6.29 ± 2.728	23.12 ± 2.607	10.34 ± 2.645
	30-49	189	6.60 ± 2.362	22.76 ± 2.636	10.89 ± 3.141
	50-69	52	6.79 ± 3.108	21.75 ± 2.700	11.35 ± 3.016
	>70	4	7.00 ± 2.944	20.00 ± 2.708	13.25 ± 1.708
	P value		0.593	0.003*	0.045
Race	Chinese	179	6.68 ± 2.576	22.09 ± 2.550	10.37 ± 3.173
	Indian	46	6.57 ± 3.270	23.04 ± 3.076	11.13 ± 3.131
	Malay	153	6.29 ± 2.403	23.38 ± 2.518	11.05 ± 2.535
	Other	6	7.50 ± 3.146	22.17 ± 3.125	13.17 ± 3.817
	P value		0.439	<0.001*	0.027*
Marital status	Single	143	6.50 ± 2.737	23.04 ± 2.575	10.50 ± 2.656
	Married	237	6.52 ± 2.545	22.51 ± 2.719	10.93 ± 3.123
	Other	4	7.50 ± 1.915	23.50 ± 2.887	11.75 ± 3.594
	P value		0.751	0.149	0.313
Education level	Secondary school	121	6.47 ± 2.422	22.19 ± 2.498	10.07 ± 3.301
	College	64	6.25 ± 2.570	22.53 ± 2.588	10.80 ± 2.890
	University	176	6.89 ± 2.659	23.43 ± 2.560	11.18 ± 2.627
	Other	23	4.70 ± 2.566	20.65 ± 3.009	11.39 ± 3.187
	P value		0.001*	<0.001*	0.010*

*P < 0.05 is significant - t-test used for occupation; ANOVA used for age, race, marital status, education level, employment status and monthly income.

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Based on the results of post-hoc Tukey tests, secondary school and university-educated women had significantly higher mean knowledge scores than women with no formal education. Self-employed women had a significantly higher mean knowledge score than housewives.

The mean attitude score of women aged 18-29 were significantly higher than women aged 50-69 (P=0.008). Post-hoc study shows no significant difference between races and practice scores although the overall P-value is 0.027. The mean attitude scores of women with no formal education were significantly lower than women with secondary school, college and university education (P-value of 0.044, 0.015 and <0.001 respectively). Women with university education also had significantly higher attitude scores than secondary school educated women (P<0.001).

Regarding practice towards osteoporosis, the secondary school-educated women had significantly lower mean practice scores than university-educated women (P=0.008). Women with monthly income less than RM 1000 had significantly lower practice scores than those with monthly income more than RM 5000 (P=0.019).

Tables 3, 4 and **5** reveal the results of Chi-square analysis with Fisher's exact test to test the relationship between race, marital status, employment status and KAP categories. Knowledge and practice categories were shown to be no relationship with race, marital status and employment status. However, there is an association between races and attitude categories.

Table 3 : Chi square analysis with Fisher's exact test to test the relationship between race, marital status, employment status and knowledge categories.

Knowledge					
Characteristics		**Frequency**			**Fisher exact**
		Good	**Moderate**	**Poor**	
Race	Chinese	3	42	134	0.094
	Indian	2	12	32	
	Malay	2	25	126	
	Other	1	1	4	
Marital status	Single	5	31	107	0.249
	Married	3	47	187	
	Other	0	2	2	
Employment status	Employed	4	44	167	0.055
	Self-employed	2	17	34	
	Unemployed	0	5	23	
	Housewife	0	8	56	
	Other	2	6	16	

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Table 4 : Chi square analysis with Fisher's exact test to test the relationship between race, marital status, employment status and attitude categories.

Attitude					
Characteristics		**Frequency**			**Fisher exact**
		Positive	**Moderate**	**Negative**	
Race	Chinese	15	136	28	0.003*
	Indian	9	32	5	
	Malay	27	119	7	
	Other	1	4	1	
Marital status	Single	24	109	10	0.159
	Married	27	179	31	
	Other	1	3	0	
Employment status	Employed	26	168	21	0.802
	Self-employed	9	38	6	
	Unemployed	6	20	2	
	Housewife	9	46	9	
	Other	2	19	3	

*P value<0.05, significant.

Table 5 : Chi square analysis with Fisher's exact test to test the relationship between race, marital status, employment status and practice categories.

Practice					
Characteristics		**Frequency**			**Fisher exact**
		Good	**Moderate**	**Poor**	
Race	Chinese	33	54	92	0.076
	Indian	11	18	17	
	Malay	27	58	68	
	Other	4	1	1	
Marital status	Single	21	51	71	0.210
	Married	52	79	106	
	Other	2	1	1	
Employment status	Employed	43	66	106	0.369
	Self-employed	15	21	17	
	Unemployed	5	11	12	
	Housewife	9	23	32	
	Other	3	10	11	

Knowledge and attitude towards osteoporosis were correlated with practice using Pearson's correlation test. Table 6 reveals that knowledge of osteoporosis was weakly positively correlated with osteoporosis practice ($r=0.241$, $p<0.001$) among the participants. The participants' attitude towards osteoporosis was weakly positively correlated with their practice towards the prevention of osteoporosis ($r=0.189$, $p<0.001$).

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Table 6 : Pearson correlation between knowledge and practice as well as attitude and practice towards osteoporosis among Malaysian women.

Variables	r	p
Knowledge vs practice	0.241*	<0.001
Attitude vs practice	0.189*	<0.001

*P value<0.05, significant.

This study reveals that Malaysian women had low knowledge, moderate attitude and low practice regarding osteoporosis. Knowledge of osteoporosis was shown to be associated with education level, employment status and occupation. Attitude towards osteoporosis was associated with age, race and education level. Practice against osteoporosis was associated with education level, occupation and monthly income of the participants. The study also found that both osteoporosis knowledge and attitude were significantly positively correlated with the practice of Malaysian women. People with good knowledge and a positive attitude towards health will be more likely to engage in health-related practice and preventive measures(7).

The study shows a low level of knowledge towards osteoporosis among participants. This result is in line with a previous study which resulted in poor osteoporosis knowledge among perimenopausal women in Vietnam (25) and Turkey (26). This contrasts with a recent study among middle-aged Chinese in Malaysia which shows moderate osteoporosis knowledge(7). The difference could be explained by the different study population, cultural difference and sample size of the studies.

In the current study, only 11.5% of women were aware that osteoporosis is a silent disease. Another recent study in Malaysia showed that 40.9% of the subjects were aware that osteoporosis does not cause symptoms such as knee pain. This may be due to the participants were confused between osteoporosis and osteoarthritis (7). The misconception should be addressed to the public. Besides, 54.4% knew that not getting active in regular exercise increases the risk of osteoporosis. 42.2% knew that early menopause is a risk factor for osteoporosis. Higher percentages were found in other studies: 90% of women in New Zealand (27) knew that regular exercise is a preventive measure; 88% of women in America(28) knew to be menopausal increases the risk of getting osteoporosis.

In the current study, knowledge was not associated with the age of the participants. This is different from previous studies in Vietnam (25) and Saudi Arabia (29) which reported level of knowledge decreases with age. Women subjects who finished high school and university scored higher knowledge scores than women with no formal education. It is known that education can affect one's ability and practice to access and interpret health knowledge and information(29, 30). This result is in line with previous studies in the USA(31)and Saudi Arabia(15). Subjects with healthcare-related occupations had better osteoporosis knowledge than others with non-healthcare related jobs. This result is in agreement with a study in Vietnam, with nurses scored higher knowledge than others(25). It may be due to their exposure to more healthcare-related problems. The monthly income of subjects shows no relationship with osteoporosis knowledge.

The mean osteoporosis attitude scores were associated with age, races and education level. Women subjects aged 18-29, Malay and had higher education were shown to have more positive attitudes towards osteoporosis. Furthermore, the classifications of attitude are associated with races of subjects. More Chinese women had a negative attitude regarding osteoporosis compared to Indian women and Malay women. This may contribute to the prevalence of hip fractures in Malaysia in which 63% were Chinese according to a survey in 2007(11).

In the study, results indicate that Malaysian women had a poor level of practice towards the prevention of osteoporosis. This finding is in line with similar previous studies in Malaysia and Saudi Arabia which showed a low frequency of preventive activity among females(7, 15). In our study, 32.0% of women exposed to the sun every day compared to only 6.8% of women had no exposure to the sun. This may be due to the tropical weather all year round in Malaysia and the participants' job characteristics that expose them to the sun. Exposure to the sun at an appropriate time is essential to obtain vitamin D which is good for bone health. Besides, only 18.5% of the participants had a history of BMD tests. However, a lower percentage was found in another study which is 14% in Saudi Arabia(15). 41.1% of women subjects had a history of taking calcium supplements. This is lower compared to a study in Saudi Arabia which showed 55% of females had a history of calcium supplement intake.

In the study, there is no significant association between the level of practice towards osteoporosis and age. Another study also found out that practice did not differ by age(15). There is a significant relationship between preventive practice and level of education. University educated women had higher mean osteoporosis practice scores than high school educated women. Women with healthcare-related occupations also had higher practice scores than those with non-healthcare related jobs. Subjects with monthly income less than RM 1000 had

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significantly lower mean osteoporosis practice scores than those with monthly income more than RM 5000. It is perhaps because people with higher income have more access to calcium-rich foods, physical exercise, BMD testing and calcium supplements.

Pearson's correlation shows both osteoporosis knowledge and attitude are correlated with practice to prevent osteoporosis. Subjects with inadequate healthcare knowledge and negative attitude tend to have a lower level of practice to prevent diseases.

Limitations **:** This study was conducted only in Klang Valley which includes the capital city, Kuala Lumpur and the surrounding area. The results may be different in other regions of the country that are not covered. The convenient method of sampling may increase the risk of selection bias.

4. Conclusion

The study showed that Malaysian women had inadequate knowledge, moderate attitude and low level of practice towards osteoporosis. This study could serve as a stimulant for policymakers to increase the education and awareness of osteoporosis among the public especially younger women. Further researchers can also assess the causes of low levels of osteoporosis knowledge and practice among Malaysians. Furthermore, the practice against osteoporosis among high-income participants was higher than low-income. This indicates that poverty should be addressed in Malaysia in order to improve bone health and to prevent osteoporosis.

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Conflict of Interest : The authors of this paper declare no conflict of interest.

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Barriers and Enhancers of Medication Error Reporting Among Hospital Pharmacists, a Qualitative Exploration

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Abstract

Reporting the medication errors (MEs) will help avoid the recurrence of the errors. The purpose of this study is to explore the enhancers and barriers of ME reporting among Malaysian hospital pharmacists. A qualitative study using in-depth interviews of 18 hospital pharmacists was conducted. Six themes and 29 codes were identified. Most of the participants agreed that medication errors were underreported in Malaysia. The main determinant of medication error reporting is perceived to be the severity of the error. Other themes include reporting system, organizational factors, reporter's burden, provider-related factors and benefit from reporting. The confidentiality of the reporting system and reporting culture in the hospital are other factors that affect MER. Huge paperwork increases reporter's burden thus hinders ME reporting. Simplifying the process of reporting, protecting the confidentiality of the process and separating MER from staff performance evaluation are the most important interventions to improve the MER rate.

Keywords : Hospital pharmacist. Medication error reporting. Medication incidents. Patient safety

1. Introduction

Medical errors have been estimated to be the third leading cause of death in the USA in 2013 with over 400,000 deaths hospitalized patients. Medication errors (MEs) were among the most common types of medical errors, harming about 1.5 million people every year and were the main contributors to adverse events to hospitalized patients (21). It imposed substantial costs between US$ 6 billion to US$ 29 billion per year .

The primary purpose of medical error reporting is to maintain healthcare providers' responsibility for performance and to produce or generate new knowledge and improve patient safety. MER allows the errors to be corrected before serious harms occur (2).

In a study from the UK, the prevalence of prescribing errors was around 9%, wherein the 19 hospital's incident reporting system, less than 0.2% of the detected prescribing errors were voluntarily reported . In the USA, direct observation of medication administration in 36 hospitals revealed an 11.7% error rate compared with just 0.04% for errors detected through the incident reporting scheme (5). The problem was probably even more serious, with estimates of underreporting of events ranging from 50% to 96% annually (7237).

According to a study in Malaysia, 17357 MEs (16%) were reported and reviewed from January 2009 to December 2012, of which 92.1% was reported by pharmacists, primarily from public-funded hospitals (12).

According to a study that included 12 Ministry Of Health (MOH) primary care clinics in four states of Malaysia, the most common clinical management errors (41.3%) were medication errors (1). From 2014 to 2015, there were 3,526 MEs and 248,307 near misses reported to the Patient Safety Unite of the Ministry of Health .

The attitudes and perceptions of doctors, nurses, midwives and other healthcare professionals towards medication errors in healthcare have been extensively studied. However, despite their key role, there were only limited publications about the pharmacists' attitude, perception, and practice of ME reporting (1).

A study investigated the perceptions and attitudes of Malaysian healthcare professionals towards medication error reporting in primary care clinics, but no study has been conducted to address the same issue among pharmacists, specifically (12).There is a small qualitative study with four participants in a single hospital in Miri Hospital, Sarawak that focuses on the causes of medication errors from the viewpoint of hospital pharmacists.

Considering that medication errors are among the most common medical errors with a high global burden, and at

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the same time, ME reporting rate is low, investigating the barriers of ME reporting matters. ME reporting is essential and important to minimize the reoccurrence of such errors and improve patient safety. Therefore, the barriers should be identified by understanding hospital pharmacists' perceptions and attitudes toward ME reporting.

2. Materials and Methods

This qualitative study uses semi-structured in-depth individual interviews. Each interview planned to last about one hour. More interviewees were invited until saturation was achieved. The term 'saturation' meant the last few interviews contain no new point to improve the targeted exploration. A thematic framework was drafted based on prior understanding. However, it was amended after data collection from participants. Purposive sampling was conducted. The participants were recruited from different departments of a variety of public and private, large and small hospitals. To improve the validity and reliability of the study, the recruited hospital pharmacists were selected purposely with diverse working experience. Recruitment of participants stopped after saturation reached, which meant further interviews did not change the current themes and codes.

All practicing Malaysian hospital pharmacists with at least one year of working experience were eligible for this study. The participants were invited through social media, personal and professional connections. Some of the participants were in the researchers' social networks, in advance and were invited to take part, as long as they fulfilled the eligibility criteria. They were invited by electronic mails or messages through the online means of communication.

The interviews were carried out by the first author who attended a workshop to learn the qualitative research techniques, at convenient places such as café, home or workplace, based upon the agreement between interviewer and interviewee.

A semi-structured interview guide was adopted from previous studies with some modifications to address the local requirements (Appendix I) (12)(1). The duration of the interview was around 45 minutes to one hour, depending on the situation. The interviews were all in English. The field notes were taken in real-time during the interview.

The procedure of data analysis was started with the verbatim transcription of the interviews. Next, in the familiarization stage, the audio recording of each interview was listened to whilst the transcription was being read. The researcher then examined a few transcripts line by line and assigned codes to denote particular meaningful segments. This coding combined both deductive and inductive approaches. The codes were grouped into categories or themes. These formed a working analytical framework that was based on the data and a prior understanding of the literature.

The framework was refined further to improve clarity, reduce ambiguity and to produce the final thematic framework as the process went further.

Subsequently, the data was charted into the framework matrix which involved summarizing data by category for each transcript. The matrix enabled the researchers to make final conclusions regarding the rich data (1).

Ethics approval was obtained from Medical Research Ethics Approval (MREC), Ministry of Health, Malaysia.

3. Results and Discussion

Table 1 shows the characteristics of the participants (n = 18) comprised of hospital pharmacists from different departments such as drug information service (DIS), in-patient pharmacy, out-patient pharmacy, as well as clinical pharmacists from public and private sectors. Two invited persons refused to participate, one of them because the supervisor asked not to take part in any interview and the other one insisted on no voice recording. The number of years of experience ranged from 22 months to twelve years.

Table 1: Characteristics of Participants

Practice Setting	**No of participants (Private sector)**	**No of participants (Public sector)**
In-patient Pharmacist	2	3
Out-patient Pharmacist	1	3
Clinical Pharmacist	1	7
Drug Information service	-	2
Duration of practice		
1 to 3 years	8	
≥3 to 5 years	8	
≥5 to 7 years	1	
≥7 years	2	

Six themes were developed by the 2 authors of this article, primarily based on the frequency of the relevant words, after 18 interviews with hospital pharmacists were conducted. Table 2 outlines the themes with associated codes. The number of pharmacists who responded to each code was recorded.

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Table 2 : Thematic Framework

Theme/Category	Codes	No. of participant responded
Nature of error	Severity of outcome	13
	Type of drug	3
	Frequency of errors	2
	unimportant errors	1
	Repetition of errors	2
Reporting Time	Reporting mode • MERS (online / paper-based) • QAP-1 form • Incident reporting • verbal	 13 2 1 5
	Confidentiality	16
	Reporting form	10
	Targeted reporting	1
Organizational factors	Push factor	4
	Feedback	18
	Maintain the professional relationship	9
	Maintain a reputation	4
	High turn-over rate	2
	Reporting culture	
	Blaming culture	7
	The concern of superiors' reaction	1
	The concern of repercussion from patient	4
	The concern of the colleague's reaction	1
	The concern of confidentiality	3
	The concern of getting a low mark in annual performance appraisal	7
Reporter's burden	Overlapping reporting	3
	Workload pressure	6
	Shortage of staff	1
	Huge paperwork	7
Provider related factors	Unclear	4
	Role in reporting	3
	Responsibility	7
	Routine task	3
	Personal fear	5
	Laziness	2
benefit from reporting	Change in practice	16
	Prevent reoccurrence	17
	Self-protection	1
	Vigilance	14

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In general, the majority of the hospital pharmacists understood the definitions of medication error and the existence of the medication error reporting system.

All of the participants agreed that medication error reporting brought benefits to patients and the healthcare system in the hospital. However, the majority of the participants agreed that the underreporting issue occurred in the hospital. 13 out of 18 participants agreed that the severity of the error outcome was the deciding factor for medication error reporting. According to the participants, multiple reporting systems were available at different settings, Medication Error Reporting System (MERS) was the most widely used.

Nature of error

Nature of error was the first theme, identified from these interviews. 13 out of 18 participants agreed that the nature of error affecting their decision to do the medication error reporting. The majority of the participants agreed that the severity of outcomes was the main factor to decide if the medication error should be reported. Besides that, the type of drugs, frequency of errors, the importance of errors and repetition of errors also affected reporting decisions.

In case the medication error brings no or mild harm to the patient, less likely it to be reported:

"If no harm to the patient, I will not report. Just inform the person that… they did errors, so that next time they will not do the error again." (DIS pharmacist, Interviewee 11)

Reporting system

The majority of the participants were aware of the methods to do the ME reporting, however, some of them were confused due to the multiple reporting modes available in their practice setting. Confidentiality issues, reporting form and targeted reporting in their setting were among other concerns of the interviewees.

MERS, either online or manual, QAP-1 form, CP3 form, Incident Reporting and finally verbal report to the superior or person-in-charge are of those different ME reporting systems.

Most of the participants stated that MERS was the most commonly used reporting mode in the hospital setting:

"We will fill up a medication error form. After that, we will send it to our drug information center and then they will have… they will help us to key in the data into the MERS system." (In-patient pharmacist, Interviewee 14)

Although confidentiality was respected in most of the hospitals, in some of the settings the identity of the reporter was not protected. This might affect the number of ME reports, according to some of the interviewees:

"People can recognize your handwriting, people see you writing. If you have an online system, maybe after work, you go back to your house, you can at least just submit it. Yes, so if the reporting is more invisible." (Out-patient pharmacist, Interviewee 8)

10 out of 18 participants said that the reporting form was tedious to fill out and too lengthy. Majority of them said a simplified reporting form or using an application would encourage them to do more reporting:

"Just that maybe we can simplify the process of reporting. So the people will actually report it." (In-patient pharmacist, Interviewee 14)

Organizational factors

There were a few organizational factors that affected the behavior of medication error reporting. Pushing factors from other professionals or superior and useful feedback encourage medication error reporting. In contrast, factors like blaming culture, concerns about maintaining the relationship with other professionals, maintaining the reputation and annual performance appraisal hinder medication error reporting.

Practitioners learn from the feedback in order to prevent the recurrence of the same medication error and to gain some new knowledge.

"We have email and in this time, we have all those (like) WhatsApp, we have a lot of groups, normally they will notify us by those kinds of channels." (Clinical pharmacist, Interviewee 16)

Some participants said they tried to maintain a good relationship with other healthcare professionals and their colleagues, therefore they didn't report some of the MEs as long as the errors didn't bring any harm to the patient:

"They will feel like we are reporting them, then is something like not good, like complaint themsometimes they will feel like… like revenge… revenge or what." (Out-patient, Interviewee 6)

The reporting culture in the practice setting is of importance and blaming culture, followed by the concern of getting a low mark in annual performance appraisal were participants' concerns. Some of the participants

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mentioned considerations about their superior, patients, colleagues and others, too:

"I would say like staff, they are quite… sometimes they quite resist of reporting any errors to us, which is because … they afraid they will be punished." (Out-patient pharmacist, Interviewee 6)

"... whenever somebody did an error, no matter is actual error or near-miss error… even near-miss error that is detected before… before actually dispensing prescription, they want to like so-called minus marks." (Out-patient pharmacist, Interviewee 4)

Reporter's burden

For some medication errors, participants said they were required to fill out more than one reporting form. This increased the burden for the reporters.

Most of the participants said they had a heavy workload in the practice setting, so it was difficult for them to do the reporting. If they wanted to report the medication errors, it actually increased their workload:

"We are busy from 8 until… until lunchtime. After lunchtime, the staff nurse will non-stop calling us, so I think is a harsher to… actually to increase our workload." (In-patient pharmacist, Interviewee 18)

Many participants stated that they needed to do a lot of paperwork to report the medication errors, they needed to provide lots of information for root-cause analysis, investigation and so on:

"I would say first, sometimes working in the government hospital, the pharmacist can be very busy, and the workload can be very high, so er… reporting all these requires a lot of paperwork and sometimes we even need to key into the computer system. So erm… it actually encourages more underreporting because of lacking time." (Out-patient pharmacist, Interviewee 8)

Provider-related factors

Referring to the attitude of the hospital pharmacists towards MER, this study came to know that most of the participants took medication error reporting as their responsibility. They perceived that medication error reporting can improve the patient's safety. However, some said they knew some coworkers who did not care and refused to do the reporting:

"Happy or not, it's a responsibility of reporting it. Because ultimately is about patient safety so no matter how, I think it's very important to report the error." (Out-patient pharmacist, Interviewee 4)

"But there are also staff who just don't care." (Out-patient pharmacist, Interviewee 6)

Personal fear of own committed error reporting was one of the factors with a negative impact on medication error reporting.

"let's say if you do something wrong, there will be sure you will feel afraid yeah, you know boss is going to scold me this and that…" (Out-patient pharmacist, Interviewee 9)

Benefit from reporting

All participants believed that medication errors reporting brings benefits to the patients as well as the healthcare system. The majority of them stated that reporting had successfully prevented the reoccurrence of similar errors. They strongly believed that medication error reporting can increase the alertness and awareness of practitioners. One of the participants considered reporting as self-protection.

According to the participants, root-cause analysis of occurred errors had been done with corrective measures such as tall-man lettering, changing of the arrangement of the medications in their practice setting to prevent the same errors from occurring again:

"We will make sure the two different medications, we put in a far place, farther place, or else we, we do tall man lettering..." (Out-patient pharmacist, Interviewee 2)

"For medication error, I feel the reporting is important because it will alert the staff, okay, to… to be more cautious, to prevent the same error from happening again." (Out-patient pharmacist, Interviewee 6)

This study revealed that Malaysian hospital pharmacists are well aware of the existence of medication errors reporting in their settings. They viewed this system positively, with benefits to the patients and the healthcare system by improving the patient's safety. This finding, as well as the underreported medication error reporting, are in accordance with other study findings, especially those studies from Malaysia that estimated MER as low as 16% (12) or 9% in the UK . However, these statistics may be argued, as direct observation of medication administration in 36 hospitals in the US revealed an 11.7% error rate compared with just 0.04% for errors detected through the incident reporting scheme (5).

The severity of the outcome of the error was the main factor to influence their decision to report. 13 out of 18 participants emphasized on this point. It seems the barriers would not hinder reporting if there is an actual severe

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medication error. This is in line with other studies (12)(8)(6)(1). This was because they strongly believed that medication error can be prevented by reporting medication errors.

In contrast, the hospital pharmacists are reluctant to report the actual medication errors if there is no harm or the harm to the patient is mild. This finding was parallel with previous studies which were published earlier (1)(4)(12).

Regarding the near miss medication errors and medication errors that impose mild or no harm to the patient, our finding is also the same as previous studies (12)(1); the error might be reported only if it is being occurred repetitively in their practice setting.

There are multiple types of medication error reporting platforms available in Malaysia. Different hospitals use different reporting platforms. The examples in government hospitals include MERS (either online or physical manual form), QAP-1 form, incident reporting and verbal reporting to the superior. Hospital pharmacists do not feel comfortable with the existence of multiple reporting platforms, as it is reported to be confusing (1). MERS was the most commonly used platform among the participants of this study. This finding is different from the previous publication where the participants use QAP and PF, majorly and perceived MERS as a duplicate task. This different finding may be primarily due to the difference in the participants' profession; ours is hospital pharmacists, whereas the previous study recruited other health-care providers in primary care clinics (12). The other reason for the observed difference could be the dominance of hospital pharmacists from the government sector (14 out of 18) in this study that warrants the usage of the national medication error reporting system. In some situations, two different reporting forms needed to be filled for a case which obviously increased the reporter's burden and hinders ME reporting.

According to our participants, although there is no intentional plan to disclose the identity of the reporters by the hospital management, the existing procedure and system of MER cannot completely protect the confidentiality of the report. It was believed that the number of medication errors reporting would have been increased If the confidentiality of the reporting could be maintained. The previous Malaysian study also concluded the same point (12).

10 out of 18 of the participants stated that the reporting forms for ME reporting were too cumbersome and time-consuming, which is in line with previous studies (12)(1). This was considered a major barrier to medication error reporting. The reporting form was complained to be very tedious, the reporting process was very lengthy, and a lot of information was required to fill out in the medication error form. It was troublesome and needed to spend a lot of time.

Of all organizational factors, perhaps the feedback on their reported medication errors is the main factor that affects the decision of pharmacists to actively report medication errors . Positive feedback enhanced or encouraged pharmacists to do more reporting in the future. Lack of feedback on the reported medication errors demotivated the pharmacists to do the reporting, on the other hand. This finding is consistent with the other studies which reported the demotivation after lack of getting feedback (1)(12). Demotivation was due to the perception that no actions were taken, or no changes happened in their practice to prevent similar medication errors from happening. Conversely, feedback on medication errors motivated the pharmacist to do the reporting. This was because they knew their work was appreciated and they did something to improve patient safety and healthcare system in their practice settings.

Another barrier to MER, based on our findings, is the concern about maintaining a good relationship with the other healthcare professionals and maintaining a harmonious working atmosphere. They had specific anxieties about the effects of reporting on interprofessional working relationships with other healthcare providers. The Same concerns have been reported by others (1). This problem can be relieved by a continuous convincing education on the importance of MER in one hand and correcting the blaming culture in case of the occurrence of medication errors, on the other hand. Around half of the participants agreed that there was a blaming culture in their practice setting and this was a barrier to MER. This finding is consistent with other studies that stated the same findings (2)(1). They were afraid to do the reporting especially if they were the ones who involved in medication errors. This is probably because they believed that the culture of blaming individuals was still present dominantly in the system. They were afraid they will be punished on their committed medication error or disciplinary action will be taken against the person who committed an error (3). The culture of 'blaming on the system' was more successful in motivating the people to do the reporting (12).

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The concern of getting a low mark in annual performance appraisal was a major barrier to medication error reporting. This finding was in line with other studies that stated they feared that these reports somehow would affect their annual performance appraisal (12). Annual performance appraisal, reflected in Key Performance Index (KPI), normally affects the staff promotion, salary increment, and bonus. In some of the settings, it was stated that the reported medication error does not affect their performance. However, in most of the hospital settings, they had the target and key performance index (KPI) that could be affected by MER. This hindered the pharmacists or co-workers from reporting. The impact of medication error on their KPI in the government sector is apparently not as big as in the private sector.

MER perceived as a burden if the reporting process was not simple and required too much extra work or time . This tedious process increased paper workload and it hindered medication error reporting (12). They needed to spend a lot of time to do these procedures in order for them to complete the form. The overlapping reporting requires more time and effort from the reporter to complete all the reporting. (192)(1).

The responsibility of a pharmacist to improve medication safety is an enhancer for medication error reporting. In this context, the pharmacist takes the responsibility of dispensing the medication properly, and to make sure that the patient receives a proper medication treatment. Medication error reporting prevents the reoccurrence of the mistake, thus improves patient safety. In some studies, it is stated that the healthcare providers' responsibility cannot be ignored and not shirking from their responsibility (1).

Personal fear of the consequences of reporting has been proposed by other researchers. People may feel fearful to report the medication error, especially when the error is committed by themselves. Such feelings may be due to the blaming culture, litigation and disciplinary procedures or their performance rate (2). They may be worried about how their colleagues thought about them or the patient's repercussion. The interesting point, however, is that personal fear may not deter a pharmacist to report where the error is severe. Probably the fear of serious harm to the patient overweighs the personal fear.

There is a general agreement among the participants that the main benefit of MER is to prevent reoccurrence in the future and thus improved patient safety. Through medication error reporting, the awareness of the medication error will be raised by providing some feedbacks such as briefing, memo or bulletin in their practice setting, this makes the colleagues more alert in the future. However, the job will be accomplished when a root-cause analysis is conducted, and the causes of the error are identified. Appropriateness of any solution would be dependent on a proper root cause analysis.

4. Conclusion

Despite the availability of several different methods of medication error reporting, the rate of reporting is still low. The diversity of reporting systems may not enhance the MER if multiple methods are being deployed in one single center. At the same time, the procedure of MER should as simple and quick as possible. The recent attempts of using software and applications can be evaluated to assess the impact of digitalization and online services on the rate of MER, not only in severe cases but also in low risk and near-miss cases. Whatever the method is, the confidentiality of the process should be guaranteed by the healthcare managers. The managers should also avoid blaming the reporters and separate the performance evaluation from MER, where they put maximum effort into correcting the system and addressing the error by proper root cause analysis. Finally, it is necessary to educate the healthcare practitioners about MER and conduct continuous reminding plans for them to keep the patients' safety as the priority and practice MER with no connivance.

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Malaysian Community Pharmacists' Knowledge, Attitudes and Practice Toward Vaccination and Immunization; A Cross-Sectional Study

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Abstract

Vaccine hesitancy is a serious problem that has been increasing in the past few decades. The contribution of community pharmacists in vaccination was found to have a good impact on immunization rates in some countries. This study aims to investigate the Malaysian community pharmacists' knowledge and attitude toward vaccine-related services, as well as the enhancers and barriers of the contribution of the community pharmacists in public immunization services. A cross-sectional study was conducted among Malaysian community pharmacists. A pre-validated questionnaire was collected from 273 community pharmacists by using online and face to face approaches, from August to October 2019. Overall, 80.2% of the participants' level of knowledge scores were moderate. The knowledge score on the influenza vaccine was higher than the other vaccine types. 61.2% of the participants were willing to contribute to vaccine administration services by giving priority to administering emergency (influenza pandemic) (61.9%), travel (43.6%) and influenza (30.8%) vaccines. 35.9% of the pharmacists counseled adults for vaccines and 35.6% promoted routine immunizations of any type for adults. The barriers to pharmacists-led immunization were the need for formal certification (93.8%), extra education/training to administer vaccines safely (89.7%), reimbursement concerns (69.6%), patients' perspective of receiving vaccines by pharmacist (63.4%), the average waiting time for immunization services (52.8%), time needed for professional development and training (50.5%) and costs needed for professional development and training (49.5%). Despite the legal and administrative limitations of practicing vaccination by community pharmacists, the overall vaccination-related knowledge of the participants is moderate. The majority of the participants were willing to contribute to vaccine administration services. Further education and training campaigns along with the financial support are needed to overcome the barriers which hinder their contribution to the immunization programs.

Key words : Vaccination.Community Pharmacy. Knowledge, attitudes and practice. Health policy

1. Introduction

In line with the necessity of increased contribution in public health, the Malaysian Pharmaceutical Society (MPS) has recently started a campaign for the involvement of community pharmacists in government vaccination and immunization programs (1). This is after prior MPS efforts in 2014 (2) and initial agreements between MPS and Malaysian Ministry of Health in 2015 and 2016, but later the Ministry did not execute the plan (1). Malaysian Medical Association (MMA) afterwards reflected and described it as 'counterproductive' (3). This particular extended pharmacy service is now in the center of some controversies and conflicts.

Vaccine hesitancy has been increasing in the past few decades, probably due to the anti-vaccine propaganda via the internet and social media in many countries. Several reports from western countries (4-6) shown the problem is serious enough to be addressed by health authorities. In Malaysia also, it is reported that the number of parents with children aged below two years refusing vaccination increased from 470 cases in 2013 to 1292 cases in 2014 (7). Re-emergence of diphtheria and measles with death tolls since 2013 (8) is now a major concern for the Ministry of Health. This situation warrants maximum utilization of healthcare providers to enhance the process of immunization in the whole society.

Community pharmacists are in the frontline of the healthcare system and most readily accessible healthcare providers. Compared with other healthcare providers, community pharmacies offer extended opening hours, more convenient locations and access to professional knowledge and expertise without requiring an appointment (9). The long history of the contribution of community pharmacists in vaccination and immunization programs in the western countries strongly supports the idea of the involvement of community pharmacists in

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dispensing and administration of vaccines. The question is how prepared the Malaysian community pharmacists are for such an extended pharmaceutical care service.

In line with the policy of increasing accessibility to immunization services which is considered as a key element in the prevention of many serious infectious diseases, community pharmacists have been actively involved in immunization services in many developed countries such as the USA (10), the United Kingdom (UK)(11), Canada (12), and New Zealand (13). It is evident that the involvement of pharmacists in immunization, whether as educators, facilitators, or administrators of vaccines, results in increased immunization rates (14).

In the USA, a study was conducted to assess the involvement of immunization-certified community pharmacists as educators, facilitators, and direct immunizers. It demonstrated the willingness of the pharmacists to be certified immunization service providers were more than 50% of the participants (15).

Another American study was conducted to assess the community pharmacists' opinions on providing immunizations. The main documented barriers to pharmacists' contribution was reimbursement concerns (66%), insufficient staffing (42%), lack of space for administration (23%), lack of physicians' support and record-keeping (22%), time for certification (10%), coast of certification (7%) and managing adverse events and liability concerns (18%). Most respondents felt fully accepted as immunization providers by patients (97%), the frequency of patients' requests for vaccine information was 23% monthly, 19% weekly and 18% daily requests. The majority of respondents (>80%) were comfortable with providing information about influenza, herpes zoster, hepatitis A or hepatitis B vaccines. In contrast, they were less comfortable with discussing pneumococcal, tetanus and meningococcal vaccines due to the lack of knowledge, reimbursement concerns and being unsure about safety in different patient populations (16).

A national survey among American community pharmacists reported that the main three barriers to the provision of immunization services were lack of time, concern for legal liability and reimbursement concerns (17).

A survey from Canada shows a high willingness among Canadian community pharmacists towards involvement in vaccination programs. The study found that 88 % agreed that the involvement of pharmacists in immunization programs would increase public access and improve rates (84 %). 68 % agreed that pharmacists should be permitted to immunize. Pharmacists indicated education, reimbursement, and negative interactions with other providers as main barriers to contribution to administering vaccines (12).

Another Canadian survey reports that 52% of community pharmacists show interest to administer vaccines, pending a legislative change. These pharmacists were more interested in administering travel (92%), flu (88%) and pandemic (85%) vaccines than regularly scheduled vaccines for adults (65%) or children (18%). Leading barriers to pharmacist-led immunization were lack of time (90%) and training (92%). The main positive determinants were identified as immunization training (95%) and adequate remuneration (92%)(18).

In a study from Saudi Arabia, 55% of the respondents expressed their willingness to administer vaccines and establish an immunization service. The remaining (45%) respondents mentioned the lack of training (75.4%) and concerns in maintaining patient safety (67.4%) as barriers to the delivery of immunization services (19).

This study will enable Malaysian healthcare policymakers and pharmaceutical professional bodies to reevaluate the contribution of Malaysian community pharmacists in public vaccination programs. We are hopeful the results of this study highlight the knowledge gap and challenges of effective CP's involvement in vaccination and immunization activities in general and in a particular manner, in addition to the assessment of CPs preparedness and willingness.

The study results might be stimulation for extra educational and training sessions for the Malaysian community pharmacists in the future that might have a positive impact on the immunization rates in Malaysia. It may also be a starting point for Malaysian pharmacy schools to help achieve conformity with national immunization objectives by training future generations of pharmacists, as they can improve immunization education and enhance the practical skills for undergraduate students of pharmacy by incorporating vaccines into their curriculum.

2. Materials and Methods

Study design

This is a cross-sectional survey among Malaysian community pharmacists, using a validated questionnaire that is adapted from a Canadian study (12). The study

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aims were to assess the beliefs, attitudes, knowledge, and practice towards vaccination and immunization among Malaysian community pharmacists.

Study settings

Community pharmacists from different states and cities (Terengganu, Johor, Kedah, Kelantan, Melaka, Pahang, Perak, Perlis, Penang, Sabah, Sarawak, Selangor, Negeri Sembilan, Putrajaya and Kuala Lumpur) participated in this study.

Study participants

All community pharmacists who practice the community pharmacy profession in Malaysia, including provisionally registered pharmacists (PRPs), were eligible. Hospital and industrial pharmacists were excluded from this study.

Study instrument

The validated questionnaire, previously published by Canadian researchers was adopted and face validation was performed by three expert academicians, followed by a pilot study, to ensure its compatibility with the local conditions. Further corrections and modifications were made, based on the feedback from experts and respondents. The questionnaire (APPENDIX II) consisted of 88 questions; 5 demographic questions, 21 questions to assess the immunization knowledge, 61 questions about the attitude toward immunization and one question about vaccine-related services (Appendix II). The first 61 questions were partitioned into six sections. The first section (general attitude) consisted of three questions regarding community pharmacists' opinions about expanding their scope of practice and adults' vaccination. The second section (specific attitude) consisted of 22 questions regarding participants' awareness about vaccines and their importance in addition to some of the possible barriers to pharmacists' involvement in immunization services. The third section (practical implications) consisted of three questions regarding participants' willingness to administer vaccines and the barriers to their contribution to vaccine administration. The fourth section consisted of 13 questions about pharmacist-related immunization practice. The 8 questions in the fifth section were about patient-related factors that impact immunization practice. The 9 questions of the sixth section were about the information and requirements in vaccine administration.

The 21 knowledge-related questions were about vaccine types, vaccines indications/contraindications, vaccines' adverse reactions, and other vaccine administration issues.

The community pharmacists' overall knowledge was categorized by partitioning the total knowledge score into three equal ranges(20); high level of knowledge if the score was between 17 to 25, moderate if the score was between 9 to 16 and low level of knowledge if the score was less than or equal to 8.

Sample Size. According to Raosoft online sample size calculator (http://www.raosoft.com/samplesize.html) assuming a 5% margin of error, 95% confidence level, 50% response rate and 3000 community pharmacies with response distribution of 50%, the required sample size for this study will be 341 respondents.

Data collection

An online survey form was created to share the study questionnaire with different networks that are linked to the Malaysian community pharmacists. At the same time, to ensure that the minimum required sample size is achievable within a preplanned time frame, manual distribution of the questionnaire was performed, on a convenience basis in Kuala Lumpur and the surrounding area in the state of Selangor, such as Puchong, Petaling Jaya, Cheras, Serdang, Gombak, Shah Alam, Klang, Subang Jaya, Sunway, Sri Kembangan, and Ampang Jaya. The data was collected from mid-August to mid-October with a total of 273 responses, both manually and online.

Data analysis

Data analysis was performed using SPSS version 22. Descriptive analysis and frequency assessment were applied to the demographic characteristics of the participants. To assess the correlation and association of the variables, Spearman's correlation was performed. To compare the means and scores of the knowledge and attitude between the groups of the participants; Mann-Whitney U and Kruskal-Wallis tests were conducted.

Study approvals

The project approval was received from Faculty Research and Scholarly Activities (FRSA), Faculty of Pharmaceutical Sciences, UCSI University. The study protocol was registered on the National Medical Research Register (NMRR) website and ethical approval was obtained via the Medical Research Ethics Committee (MREC), the Ministry of Health, Malaysia.

3. Results and Discussion

A total of 273 community pharmacists participated in the study. There was no significant difference between

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male and female participants. Since the questionnaire link was shared with community pharmacists via different platforms the authors could not stipulate how many pharmacists had reached the link. Therefore, the response rate could not be estimated due to the missing denominator.

The education level of 185 (67.8%) of the participants was undergraduate whereas 88 (32.2%) were postgraduate as shown in Table 1.

Table 1 : Demographic Data Variables

Variables	**N**	**%**
Gender n=273		
Male	138	50.5
Female	136	49.5
Qualification n=273		
Undergraduate	185	67.8
Postgraduate	88	32.2
Position n=273		
Staff pharmacists	158	57.9
Manager	44	16.1
Relief pharmacist	31	11.4
Owner	22	8.1
Clinical pharmacist	18	6.6

Regarding the participants' position, 158 (57.9%) of the participants were staff pharmacists, 44 (16.1%) managers, 31 (11.4%) relief pharmacists, 22 (8.1%) owners, and 18 (6.6%) clinical pharmacists. In terms of the primary state of practice, 121 (44.3%) of the participants were practicing in Selangor, 77 (28.2%) in Kuala Lumpur, 24 (8.8%) in Sarawak, 20 (7.3%) in Sabah. The primary practice state of the remaining (11.4%) was Johor (4.4 %), Putrajaya (2.9 %), Perak (2.9%) and Labuan (1.2%).

Regarding working experience, 104 (38.1%) of the participants reported 1 to 5 years, 85 (31.1%) stated 6 to 10 years, 47 (17.2%) had 11 to 20, 26 (9.5%) declared less than one year and only 11 (4.1%) declared 21 to 30 years.

In response to a question about their weekly working hours, 172 (63%) were working more than 40 hours per week, 75 (27.5%) reported 25 to 40 hours per week, 23 (8.4%) replied 11 to 24 hours per week and only 3 (1.1%) reported that they work less than 10 hours per week. Table 2 summarizes the participants' information about their pharmacy practice.

Table 2 : Pharmacy Practice Variables

Variables	**N**	**%**
State of primary practice n=273		
Selangor	121	44.3
Kuala Lumpur	77	28.2
Sarawak	24	8.8
Sabah	20	7.3
Johor	12	4.4
Putrajaya	8	2.9
Perak	8	2.9
Labuan	3	1.2
Years of working experience n=273		
1 to 5 yeas	104	38.1
6 to 10 years	85	31.1
11 to 20 years	47	17.2
Less than one year	26	9.5
21 to 30 years	11	4.1
Working hours per week n=273		
More than 40 hours	172	63
25 to 40 hours	75	27.5
11 to 24 hours	23	8.4
Less than 10 hours	3	1.1

The specific participants' knowledge about the influenza vaccine is reflected in Table 3.

For example, the necessity of immunization of pregnant women with influenza vaccine if delivery during the influenza season is expected was questioned. The table demonstrates a high level of knowledge, compared to other components of knowledge in the questionnaire.

The findings of the knowledge of the participants about the other types of vaccines are summarized in Table 4. The questions of this section were about the pneumococcal vaccine, tetanus-diphtheria vaccine, polio vaccine, and so on. The percentage of given correct answers is low compared to the influenza vaccination section.

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Table 3 : Influenza Vaccine Correct Responses

Items (N= 273)	N	%
Unvaccinated people with mild symptoms of influenza can spread the disease to others.	214	78.4
Influenza vaccine should not be given during the first trimester of pregnancy.	114	41.8
Annual influenza immunization is recommended for all health care professionals in contact with individuals in high-risk groups.	246	90.1
What it the reported vaccine-associated adverse event after immunization with influenza vaccine that contraindicates to subsequent immunization.	246	90.1
Influenza vaccination is considered safe in pregnancy.	139	50.9

Table 4 : Other Vaccine Types Correct Responses

Items (N= 273)	N	%
Pneumococcal vaccination is contraindicated for a splenic (without a spleen) patients.	59	21.6
One tetanus-diphtheria booster in adults should be replaced with one dose of tetanus, diphtheria, acellular pertussis (Tdap) vaccine.	92	33.7
Adults ≥ 65 years may receive either polysaccharide or conjugate pneumococcal vaccine.	47	17.2
Which of these are Live Vaccines?		
Oral polio	54	19.8
Measles-Mumps-Rubella	179	65.6
Varicella	108	39.6

The pharmacists were asked if there is any evidence to support an association between vaccines and multiple sclerosis; 58.5% chose "Don't know", 26.5% "False" and only 15% reported that this statement is true. The participants were questioned regarding the vaccines that could be given to individuals with an anaphylactic reaction to eggs, 58.2% of the pharmacists selected "Influenza", 32.6% "Measles-Mumps-Rubella", 21.7% "Meningococcal", 20.9% "Hepatitis B" and 11.7% "Pneumococcal".

Regarding anaphylaxis following vaccination, 56% of the community pharmacists chose "Prompt administration of subcutaneous or intramuscular epinephrine is the most important step in the management of anaphylaxis", 45.1% "Anaphylaxis occurs following approximately 1/10,000 vaccine doses administered", 30.4% "The development of an urticarial skin rash (hives) at the site of vaccine administration may occur as an early sign of anaphylaxis and should be managed as anaphylaxis" and only 6.6% "Epinephrine should be administered in the same limb as the vaccine".

The community pharmacists were asked whether their opinions should be sought when the scope-of-practice is expanded to include the administration of vaccines to adults. The majority of the pharmacist (82.5%) reported that their opinion should be sought to include the administration of vaccines among the adults and only while 13.9% neither agree nor disagree and the remaining 9.9 % disagreed agree for the above query. In response to the question of whether the provision of immunizations to adults is adequate, 40.3% of the participants disagreed, 31.9% agreed and 27.8% neither agreed nor disagreed. Regarding the importance of receiving all adult vaccines recommended by Malaysian guidelines, 83.5% chose "Agree", 9.2% chose "Neither agree nor disagree" and only 7.3% chose "Disagree". Referring to the importance of increasing the proportion of adults who receive recommended immunizations, 92.6% agree, 5.1% did not agree or disagree and only 2.2% disagree.

The participants were asked about the importance of getting annual influenza vaccine and tetanus-diphtheria toxoid vaccine every 10 years; 82.4% agree that the annual influenza vaccine is important, 11.8% didn't agree or disagree and only 5.9% reported that they disagree. Most of the pharmacists (75.1%) reported that they agree with the importance of receiving the tetanus-diphtheria vaccine every 10 years, 20.5% didn't agree or disagree and only 4.4% disagree.

In terms of risks and benefits of vaccines, 89.4% agree that vaccines produce more health benefits than health risks, 7.3% did not agree or disagree and only 3.3% disagree. 72.9% of the participants agreed that vaccines are adequately tested for safety, 25.3% didn't agree or disagree and only 1.8% disagreed. Most of the respondents (85%) agree that the serious adverse reactions to vaccines are rare.

Regarding the frequency of the vaccine-related questions, asked by patients 28.2% disagree that they are frequently asked by the patients for advice about vaccines, 46.5% agree and 25.3% did not agree or disagree. When the question was about their comfortability to answer the patients' inquiries, 77.7% agree.

The impact of the pharmacists' contribution to the

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vaccination, was assessed by respondents positively (85.4%). The participants were also asked about vaccine administration issues. 83.6% of the pharmacists agreed that pharmacists should be permitted to expand their practice to include administration of recommended adult vaccines.

The incorporation of immunization services in community pharmacy practice was also questioned, where 61.2% responded positively, 28.6% "Unsure" and only 10.3% responded "No".

When the pharmacists were asked if an immunization training or certification program was available for them which one of the immunization services they feel they apply to them, 61.9% reported "I would vaccinate in emergency situations (e.g. influenza pandemic)", 39.2% reported "I would vaccinate in a collaborative framework where a physician recommends a vaccine first and then I administer the vaccine", 41% reported "I would vaccinate in a collaborative framework where a physician vaccinated the first time and I could provide all subsequent vaccinations of the same vaccine", 30.8% reported "I would be comfortable administering influenza vaccine only", 43.6% reported "I would administer travel vaccines" and only 8.8% reported, "I would not administer vaccines".

Currently, the Malaysian community pharmacists do not administer vaccines, although they may provide some vaccine-related services. 35.9% of the participants stated that they actively counsel adults for vaccines and 35.5% reported that they don't provide any vaccine-related services.

The moderate level of knowledge in the Malaysian community pharmacists can be attributed to low contribution to immunization programs in Malaysia (1-3). This may hinder a direct exposure to the practice and subsequently affect their experience and skills. The outcome of the lack of exposure would result in suboptimal knowledge and motivation to learn about vaccines or to improve their current knowledge about different aspects of immunization (21, 22). Almost 70% 0f the respondents failed to answer the question about the management of anaphylactic reactions to the vaccines correctly. This problem can also be attributed to curricular issues in the pharmacy program in Malaysia where most of the private and public schools and colleges do not provide adequate training and education courses about vaccines and immunization programs (23). This is supported by the current study finding where only 16.9% of the respondents agreed that they had received an adequate education or training. It is not surprising that 89.7% stated that they need additional education and training to be able to administer vaccines safely. Some Canadian studies (12, 18) along with a study from Saudi Arabia (19) reported a good level of vaccine-related knowledge among community pharmacists and at the same time an adequate education and training courses about vaccines and immunization in the taught pharmacy programs. The same stipulation can be extrapolated to the findings from the US, as well (15). The involvement of American community pharmacists in vaccine administration and services undoubtedly provides a good opportunity to expand their knowledge in this field. The correlation between practicing a specific task and attributed knowledge level would be more obvious when the influenza-specific knowledge score is compared with the general vaccination knowledge in our respondents. Influenza-specific knowledge is higher, simply because the community pharmacists are being queried by clients more. The fact that 92.7% of the respondents reflect comfortability and willingness to administer the influenza vaccine is another aspect of the same concept. This is in line with a study results (16) that demonstrated American community pharmacists were more knowledgeable on influenza, herpes zoster, hepatitis A and hepatitis B vaccines as compared to their knowledge in pneumococcal, tetanus and meningococcal vaccines.

Regarding the safety of vaccines, their relationship with multiple sclerosis and specifically the link between egg allergy and influenza vaccine contraindication, the respondents' feedback shows that most of the community pharmacists are not well updated. While doubts about the link between vaccines and multiple sclerosis were expressed in the older literature (24, 25), recent pieces of evidence deny such a linkage (26, 27). Moreover, it was believed that the influenza vaccine is contraindicated or should be given with supervision and caution for patients with egg allergy (28)(29). However, recent studies show that the influenza vaccine can be given to patients with egg allergy safely (30, 31). 73.5% and 58.2% of our respondents failed to demonstrate the updated knowledge regarding the linkage between vaccines and multiple sclerosis and egg allergy, respectively.

Our findings show that the male participants have a higher level of knowledge than the female participants (mean rank 147.11 vs. 126.67, $p = 0.032$). This is correlated and perhaps explained by longer working

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experience of male participants than the female participants. Where the number of female participants in the category of 'below one year working experience' was significantly higher than the male participants (18 vs. 8, $p<0.05$), the number of male participants in the category of '11 to 20 years working experience' was significantly higher than the number of female participants (31 vs. 16, $p<0.05$).

Despite all limitations and obstacles of community pharmacy practice in Malaysia, this study shows that Malaysian community pharmacists are willing to step up their area of practice beyond the existing situation. Only 10.3% of the participants were not keen to get involved in vaccine administration and immunization programs. The fact that 83.6% of the participants agreed that community pharmacists should be permitted to administer adult vaccination shows that community pharmacists are well aware of the necessity of establishing extended pharmacy services, including vaccination and immunization services.

The participants strongly believe (85.4%) that the proportion of adults who receive recommended immunizations would increase if community pharmacists were permitted to administer vaccines to adults, due to improved access to immunization services.

The current study results are comparable with the other studies from Canada (12), the USA (15), and Saudi Arabia (19) in terms of the attitude of the participants. Similar to the American study, the majority of participants of current study mentioned improving public healthcare as their main driver of willingness toward being a certified vaccine administrator. Regarding the frequency of vaccine-related queries, 23% reported monthly, 19% reported weekly requests and 18% reported daily requests. This load of queries may justify the high level of willingness for practicing vaccine administration in community pharmacists.

Despite the positive attitude of the participants towards practicing vaccination, the knowledge gap may play a negative role in such an achievement. However, the obstacles are far beyond the sole knowledge gap, according to the participants. The study participants pointed out the time needed for professional development training, the cost associated for professional development training, reimbursement concerns (including the availability of staff and their time to provide vaccines), physician support, people's confidence and safety about receiving vaccines from pharmacists and lack of formal certification as potential obstacles (Table 5).

Table 5 : Frequent Barriers to Vaccine Administration

Item	N	%
Formal certification needed for vaccine administration	256	93.8
Reimbursement concerns (time and staff support)	190	69.6
Time needed for professional development and training	138	50.5
Costs needed for professional development and training	135	49.5
The availability of staff support	206	75.5
The average wait time for immunization services	144	52.8
Patients' perspective of receive vaccines by pharmacist	173	63.4
Patients' confidence in pharmacist ability to administer vaccines	158	57.9
The need for more education/ training for vaccines administration	208	76.2
Inadequate education/training about immunization	166	60.8

Of all those potential barriers, lack of physician support should be considered as a specific matter in the context of current pharmacy practice in Malaysia. Malaysian community pharmacists struggle for separating dispensing and prescribing has still been fruitless after decades. Seeking for other areas of extended pharmacy services, in the atmosphere of tension between community pharmacies and dispensing physicians, probably would not be welcomed by physicians. Malaysian Medical Association (MMA) reflection (3) to the Malaysian Pharmaceutical Society (MPS) attempts toward vaccination and immunization services, then was not surprising. Others have also reported the physicians' opinion that the community pharmacists should focus on their main role as medication experts to oppose their contribution in extended services such as vaccination (32).

Higher attitude scores among the participants from Selangor and Kuala Lumpur states as compared to Sarawak, Sabah, Johor, Perak, and Labuan, is probably due to the type of queries in metropolitan areas, including higher public attention to the adult vaccination issues (33,

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34). Meanwhile, the current study findings show that the willingness of pharmacists to practice vaccination is reversely correlated with working hours per week (rs = -0.124, p= 0.041).

In response to the questions regarding the barriers to vaccination practicing, the pharmacy owners obtain a higher attitude score compared to the manager or clinical pharmacists. Probably the owners are more concerned about the financial gain of this extended pharmacy service. At the same time, it can be stipulated that the clinical pharmacists, relief pharmacists and managers are more cautious in adopting new services because they have direct exposure to the patients, clients and stakeholders, so that they may understand the barriers in a more practical way than the owners.

4. Conclusion

Malaysian community pharmacists' knowledge of vaccination and immunization needs to be improved by curricular modifications in pharmacy programs, as well as conducting training and workshop sessions for practicing pharmacists. Their participants' attitudes and willingness toward involvement in vaccination programs, however, are very positive. This is an opportunity for policymakers in Malaysia to provide suitable circumstances for utilizing this huge potential for boosting public health via vaccination services by community pharmacists. Since community pharmacists from several different states participated in this study, the findings can be assumed applicable to the whole country.

Further education and training campaigns, providing financial support for the pharmacists' continuous development programs and recruiting more efforts to influence the collaborative work between the community pharmacists and the physicians or the other healthcare providers may enhance the contribution of Malaysian community pharmacists in vaccination and immunization services. Shifting from a medication-centered and dispensing-based job to a healthcare service provider profession took place once, more than 6 decades ago. The transformed roles and advancements in the function of community pharmacists in developed countries provide a doable promising prospect of extended pharmacy services, such as vaccination, in Malaysia.

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Conflict of interest

The authors of this article declare no conflict of interest.

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Knowledge, Attitude and Practice of General Public Towards Counterfeit and Adulterated Medicines : a Cross-sectional Study in Malaysia

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Abstract

Counterfeit and adulterated medicines have become a global threat. No documented benchmark and framework that can be used to evaluate knowledge and practice among Malaysian. The main objective of the research is to assess the public's knowledge, attitude and practice (KAP) towards counterfeit and adulterated medicines (CFAM) in Kuala Lumpur, Malaysia. The research is also conducted to evaluate the relationship between demographic variables and the level of KAP of Malaysian general public on CFAM and assess the opinion of public on the use of education to combat the CFAM issue. A descriptive cross-sectional study using a validated, self-administered questionnaire was conducted among public using a convenient sampling technique. This study was approved by the Medical Research Ethics Committee. A total of 387 volunteers participated in the study. Data including respondents' demographic characteristics, KAP regarding CFAM and opinion on education to combat CFAM were collected. IBM SPSS Statistic® version 20 was used to analyse the collected data.43.9% of respondents had a moderate level of knowledge towards CFAM. 54.5% of respondents showed a positive attitude towards CFAM. However, 53% of respondents demonstrated negative practice against CFAM. Occupation of respondents had a significant association with knowledge on CFAM. Highest level of education and occupation of respondents were significantly associated with attitude against CFAM. Significant associations between the highest level of education, employment status and occupations with the practice of respondents against CFAM were revealed. Overall, the general Malaysian public in Kuala Lumpur had a moderate level of knowledge, positive attitudes but negative practice towards CFAM. Although knowledge of respondents is positively correlated with attitude and practice on CFAM, the practice of respondents towards CFAM is unsatisfactory despite positive attitude on CFAM. Occupation is the most important predictors for the level of KAP of respondents, followed by the highest level of education and employment status. Health care providers and health workers are encouraged to deliver information of CFAM to the public to narrow the gap of knowledge of CFAM.

Key words : Counterfeit and Adulterated Medicines, Knowledge, Attitude, Practice, Kuala Lumpur

1. Introduction

According to the U.S. Food and Drug Administration, counterfeit medicine is defined as medicine which may contain wrong or no active ingredient (1). Counterfeit medicine includes those with right active ingredient but wrong dose or those medical products which are contaminated. Adulterated medicines are medicines that contain or are mixed with substances which may diminish the effect of medicines or jeopardise the health of consumers (2).

Before a medicinal product can be marketed in a certain country, it must be registered with local drug regulatory authority. All medicinal products must be compliant to the specifications of approval for registration of medicinal products. Responsibility of the authority is to ensure that all medicinal products conform to established criteria of quality, safety and efficacy (3). However, Counterfeit and adulterated medicine (CFAM) have become a growing threat worldwide. A systematic review reported that the prevalence of CFAM in lower- and lower-middle-income countries throughout Africa and Asia is high (4).

Developing countries are main targets for distribution of CFAM due to unaffordability of costly medications by the population (5). High demand in the market drives the infiltration of CFAM into supply chain easily (6). Therefore, most commonly counterfeited medicines are those costly medicines used for the treatment of life-threatening conditions such as cancers and infectious diseases (7). In developed countries, expensive lifestyle medicines are most commonly counterfeited, such as

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hormones and steroids pills (7). CFAM has developed differently between developing and developed country. Consequently, countermeasures to overcome CFAM should be catered for such differences between regions.

From a survey conducted in 2013, almost 5% of medicines sold in Malaysia was fake (8). Serious concern has been raised in the country on the emerging issue of CFAM. However, there are limited local studies to assess the knowledge, attitude and practice (KAP) of the general public on CFAM. Association between demographic characteristics of Malaysian with knowledge, attitude and practice towards CFAM are yet to be identified. Hence, we conducted a study in the federal territory of Kuala Lumpur, the capital of Malaysia to assess the level of KAP of Malaysian general public on CFAM and evaluate the relationship between demographic variables and the level of KAP of Malaysian general public on CFAM.

2. Materials and Methods

Study design **:** A descriptive cross-sectional study was conducted between May and August 2018 to assess the KAP of the general public towards CFAM in Kuala Lumpur, Malaysia. The study design and questionnaire were approved by the Medical Research Ethics Committee (MREC), Malaysia. (reference no: KKM.NIHSEC.P18-1673 (5)).Thiscross-sectional study followed the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) guideline (9). Given that these are anonymous surveys, the identity of the participants or their affiliated institution was not collected. Responding to the surveys by participants were considered as implied consent.

Study instrument **:** A self-administered questionnaire was developed by adaptation and modification from a previous study on counterfeit medicines to suit with local population (10).

The questionnaire was constructed in English and consisted of five sections :

A. Demographics. This section sought details of eight demographic data.

B. Knowledge about Counterfeit and Adulterated Medicines. This section contained 8 statements that the respondents used to describe the CFAM (Yes, No or Unsure).Each correct answer was given '1' point while the unsure or wrong answer was given '0' point. A sum score (from a minimum of '0' to a maximum of '8') for each respondent was then computed. The level of respondent's knowledge was stratified into poor (0-2), moderate (3-5) and good (6-8), respectively.Mean score for each item in the knowledge domain was computed. The higher the mean score (closer to 1), the better the knowledge of respondents on the respective questionnaire item.

C. Attitudes towards Counterfeit and Adulterated Medicines. This section contained 10 statements to evaluate the attitudes of respondents towards CFAM.Each positive sentence was graded as follows: "strongly disagree", 1 point; "disagree", 2 points; "neutral", 3 points; "agree", 4 points and "strongly agree", 5 points. In contrast, the negative sentences (statement 1, 4 and 7) were graded in reverse order. A sum score (from a minimum of '10' to maximum of "50') for each respondent was then computed. The respondent's attitude was stratified into negative (10-23), neutral (24-37) and positive (38-50), respectively. Mean score was calculated for each item in the attitude domain. Respondents demonstrate better attitude to the respective questionnaire item with a higher mean score (closer to 5).

D. Practice against Counterfeit and Adulterated Medicines. This section contained 5 statements to assess the practice of respondents about the genuine medicine utilisation.Responses to the positive sentences (statements 1, 2, 3 and 4) were graded as follows: "almost never", 1 point; "sometimes", 2 points; "often", 3 points; and "always", 4 points. The negative sentence (statement 5), was graded in reverse order. A sum score (from a minimum of '5' to maximum of "20') for each respondent was then computed. The respondent's practice was stratified into negative (5-10), neutral (11-15) and positive (16-20), respectively.Respondents practice positively towards items with a higher mean score (closer to 4).

E. Public advice on measures to reduce counterfeit and adulterated medicines. This section contained two questions to seek public opinion on the use of education to combat the CFAM issue.

Reliability and validity of study instrument: Reliability and validity testing of the questionnaire were performed. Content and construct validity of the questionnaire was conducted by pharmacy lecturers and subject matter experts. The questionnaire was pilot tested in two phases with a convenience sample of 60 Malaysian general public for face validity and reliability of the questionnaire. Cronbach's alphas of the final questionnaire were 0.887 (knowledge domain), 0.838 (attitude domain) and 0.781 (practice domain), respectively.

Sampling method and sample size: A convenience sampling method was adopted in this study. The sampling frame is the Malaysian general public who reside in the

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federal territory of Kuala Lumpur. Verbal verification of Malaysian citizenship of respondents was done before distributing the questionnaire. By using Raosoft® sample size calculator, the minimum sample size required (confidence interval = 95%; margin of error = 5%) was 384 for this study (11).

Study participants and data collection: All Malaysian citizens who reside in Kuala Lumpur, aged 18 or above with English literacy were eligible to participate in the study. The exclusion criterion was one who refuses to participate in the survey. The validated self-administered questionnaires were distributed to the crowds in public areas. An informed consent form was attached along with each questionnaire,participants were aware that their willingness to participate were fully respected andthe result of data analysis will be published in journal for academic purpose. Their anonymity and confidentiality were preserved.

Data analysis: The data were analysed using Statistical Package for the Social Sciences version 22.0 (12). Descriptive analysis was used to tabulate the respondents' demographics and level of KAP in frequencies and percentages. Chi-square test was employed to assess the association between the demographic variables and the KAP outcomes. Logistic regression analysis was used to determine the predictors of KAP. Multinomial logistic regression was employed for knowledge and practice whereas binary logistic regression was utilised for attitude domain. A p-value of less than 0.05 was regarded as statistically significant.Spearman's rho correlationtest was conducted to determine the possible relationship between respondents' knowledge-attitude, knowledge-practice and attitude-practice on CFAM.

3. Results and Discussion

387 questionnaires were completed and collected from the respondents.

Socio-demographic characteristics of respondents: The socio-demographic characteristics of 387 study participants are summarised in Table 1. Majority of the respondents aged between 18 and 24 years (N= 180, 46.5%), Chinese (N=237, 61.2%) ethnic, and with degree qualification (N=252, 65.1%). The mean age of respondents participated in this study is 28.13±10.11 years (range = 18-68 years old).About 50% of the respondents are employed in non-healthcare related sector, while nearly one-third have monthly income ranged from RM3,000 to RM5,000. It is also noteworthy that approximately one-third of the respondents refused to disclose their occupation and monthly income.

Table 1: Socio-demographic characteristics of respondents (N=387)

Demographic Parameters	Frequency (%)
Age (years)	
18-24	180 (46.5)
25-39	159 (41.1)
40-54	29 (7.5)
More than 55	19 (4.9)
Race	
Malay	105 (27.1)
Chinese	237 (61.2)
Indian	35 (9.0)
Others	10 (2.6)
Highest level of education	
Primary School	2 (0.5)
Secondary school	34 (8.8)
Pre-university	69 (17.8)
Degree	252 (65.1)
Master	16 (4.1)
PhD	14 (3.6)
Employment status	
Employed	222 (57.4)
Self-employed	13 (3.4)
Unemployed	17 (4.4)
Housewife	9 (2.3)
Student	123 (31.8)
Retired	2 (0.5)
Others	1 (0.3)
Occupation	
Healthcare related	98 (25.3)
Non healthcare related	188 (48.6)
Not applicable	101 (26.1)
Monthly income	
<1,000	36 (9.3)
1,000-3,000	85 (22.0)
3,000-5,000	118 (30.5)
>5,000	34 (8.8)
Not applicable	114 (29.5)

Table 1 shows socio-demographic characteristics of 387 respondents participated into this survey.

Knowledge ofcounterfeit and adulterated medicines: Majority of respondents had a moderate level of knowledge on CFAM (N=170, 43.9%) with a median score of 4.37% (N=143) and 19.1% (74) of respondents have poor and good knowledge on CFAM respectively. Breakdown of respondents' knowledge score on CFAM for the eight questionnaire itemsis shown at table 2. The results revealed that most respondents were aware that

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Table 2 : Breakdown of respondents' rnowledge, attitude and practice score on CFAM per questionnaire item

No.	Questions	Score
	Knowlegde	
1.	If there is no Meditag™ Hologram security sticker on the label/outer packaging of a pharmaceutical product, then it's a "counterfeit pharmaceutical product".	0.421 ± 0.494
2.	If there is no registration number starting with the alphabets "MAL" printed on the label of a pharmaceutical product, then it's a "counterfeit pharmaceutical product".	0.302 ± 0.460
3.	Brand and generic medicines are found counterfeited or adulterated and sold for profit.	0.522 ± 0.500
4.	Quality, efficacy and safety of CFAM are not guaranteed.	0.455 ± 0.499
5.	The problem of CFAM affects not only developed countries.	0.553 ± 0.498
6.	Medicines for treating chronic and serious diseases ('lifesaving medicines') such as heart disease or cancer can be counterfeited/ adulterated.	0.279 ± 0.449
7.	The Internet has become a major platform for circulating CFAM.	0.587 ± 0.493
8.	CFAM can be discovered in the legal medicine supply chain, that is, through licensed wholesalers and traders.	0.248 ± 0.432
	Attitude	
1.	Counterfeit drugs are not as good as authentic drugs.	3.698 ± 1.023
2.	There is high probability that the counterfeit drug doesn't work.	3.602 ± 1.041
3.	Authentic drugs are worth the money they cost.	3.452 ± 0.984
4.	I do not prefer to buy pharmaceutical products from online pharmacy/drug retailer.	3.501 ± 1.184
5.	I agree that it is important for consumers to consult pharmacist before buying any pharmaceutical products.	4.080 ± 1.026
6.	I agree that it is important for a pharmaceutical product to display Meditag™ Hologram security sticker and registration number on the label/packaging.	4.010 ± 0.979
7.	I do not prefer to buy cheap pharmaceutical products regardless the source of the products.	3.897 ± 0.968
8.	I should immediately inform my physician and pharmacist if I suspect that the pharmaceutical products are counterfeits.	3.858 ± 1.025
9.	I agree that using CFAM will put my health at risk.	3.778 ± 0.991
10.	I agree that purchasing CFAM will bring harm to economy of the country through loss of taxation revenue.	3.631 ± 0.947
	Practice	
1.	I check if there is Meditag™ Hologram security sticker on pharmaceutical product before purchasing any pharmaceutical product.	1.995 ± 0.939
2.	I check if there is registration number on the pharmaceutical product before purchasing any pharmaceutical product.	1.889 ± 0.908
3.	I inform physician /pharmacist /drug retailer if there is no Meditag™ Hologram security sticker on a pharmaceutical product.	1.744 ± 0.919
4.	I inform physician /pharmacist /drug retailer if there is no registration number on a pharmaceutical product.	1.713 ± 0.895
5.	I do not buy the pharmaceutical product that is cheaper than other similar product regardless of the source.	3.279 ± 0.775

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the internet is a major platform for circulating. Unsurprisingly, respondents demonstrated the poorest knowledge on the discovery of CFAM in the legal medicine supply chain.

Attitude on counterfeit and adulterated medicines: Around 54.5% (N=211) of the respondents showed positive attitude (score = 38-50 points) on CFAM with median score of 38. Respondents with a negative attitude (3.4%, N = 13) may feel that CFAMhave no harm on their health, whereas respondents with neutral attitude (42.1%, N = 163) have mixed opinion on harmful effects of CFAM. Among the 10 questions assessing respondents' attitude on CFAM (Table 2), most respondents agreed that consulting pharmacist before buying any pharmaceutical productis important. Interestingly, majority of respondents disagreed about authentic drugs worth the money they cost.

Practice on counterfeit and adulterated medicines: More than half of respondents (53%, N = 205) practice negatively in issues related to CFAM with a median score of 10. Only 8% of respondents (N = 31) demonstrated positive practice towards CFAM. According to Table 2, question 5 under the domain of practice ("I buy the pharmaceutical product as long as it is cheaper than other similar product regardless of the source of the pharmaceutical product.") was given the highest score among all the question in practice domain (mean score = 3.279 ± 0.775) by the respondents.

Association between level of knowledge, attitude and practice with demographic characteristics of respondents:Occupation of respondents is the only parameter with a significant association with knowledge, attitude and practice of respondents towards CFAM, as shown in table 3. The other two characteristics found to be significantly associated with attitude and practice arethe highest level of education and employment status of respondents.

Table 3 : Association between knowledge, attitude and practice with demographic characteristics of respondents

Demographic characteristics	Knowledge		Attitude		Practice	
	χ^2	*p*-value	χ^2	*p*-value	χ^2	*p*-value
Age	6.63	0.352	3.37	0.337	1.58	0.960
Race	8.65	0.059	3.44	0.323	10.72	0.080
Highest level of education	11.79	0.063	17.28	0.001*	12.11	0.050*
Employment status	11.52	0.061	4.23	0.238	12.00	0.046*
Occupation	53.22	0.000*	53.22	0.003*	17.46	0.000*
Monthly income	11.91	0.186	4.84	0.307	3.09	0.935

x^2: chi square

*: p value value less than 0.05

Predictors of level of knowledge, attitude and practice on CFAM: Table 4 shows that the occupation of respondents is the only significant predictor for the respondents' level of knowledge on CFAM. Respondents with healthcare-related occupation will likely to have a better level of knowledge on CFAM than that of non-healthcare related occupation.According to table 5, the highest level of education and occupation significantly predict one's attainment of positive attitude on CFAM. Healthcare related workers are more likely to have a positive attitude to non-healthcareelated counterpart.

As shown in Table 6, employment status, the highest level of education and occupation were found to be the significant predictors for the level of practice on CFAM of respondents. Unemployed respondents were less likely to practice neutrally compared to the student. Respondents attained postgraduate level of education were more likely to have neutral practice than the student. When comparing positive practice to negative practice on CFAM, respondents working in the healthcare-related field were more likely to practice positively on CFAM compared to non-healthcarecounterpart.

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Table 4 : Forward stepwise multinomial logistic regression analysis of factors associated with poor knowledge on CFAM

	SE	df	OR	95%CI	*p*-value
Reference: Poor level of knowledgeModerate level of knowledge vs. poor level of knowledge					
Occupation Healthcare related Non healthcare related (control group)	0.358	1	4.626	2.294:9.329	0.000*
Reference: Poor level of knowledgeGood level of knowledge vs. poor level of knowledge					
Occupation Healthcare related Non healthcare related (control group)	0.394	1	12.571	5.845:27.034	0.000*
R^2 = 0.128 (Cox and Snell), 0.146 (Nagelkerke) χ^2 (2) = 52.785 *p<0.05					

SE : standard error, df: degree of freedom, OR: odd ratio, CI: confidence interval, x^2 : chi-square

Table 5 : Binary logistic regression analysis of factors associated with attitude on CFAM

	SE	df	OR	95%CI	*p*-value
Reference: Neutral attitude **Positive attitude vs. neutral attitude**					
Highest level of education					
Pre-University	0.434	1	0.597	0.255:1.397	0.234
Degree	0.383	1	0.862	0.407:1.825	0.698
Postgraduate	0.561	1	0.166	0.055:0.499	0.001*
Secondary school (control group)					
Occupation Healthcare related Non healthcare related (control group)	0.280	1	2.285	1.356:3.849	0.002*
R^2 = 0.072 (Cox and Snell), 0.096 (Nagelkerke) χ^2 (4) = 27.557 *p < 0.05					

SE: standard error, df: degree of freedom, OR: odd ratio, CI: confidence interval, x^2: chi-square

Correlation of knowledge, attitude and practice on CFAM: There was a statistically significant positive correlation between knowledge with attitude and practice as shown in table 7.The higher the knowledge of respondents towards CFAM, the more positive the attitude and practice of respondents towards CFAM.

Education on counterfeit and adulterated medicines: Majority of respondents(48.8%, N = 189) had never heard about the term "Counterfeit and/or Adulterated Medicines" before study participation. 10.3% (N = 40) of respondents were unsure about the meaning of counterfeit medicine whereas 40.8% (N = 158) of them heard about counterfeit medicine before.Interestingly, around72.1% (N = 279) of respondents had never suspected about the authenticity of purchased medicines. As high as 75.5% of respondents (N = 292) agreed that education as part of the solution to combating/stopping CFAM. Public educationshould be part of the comprehensive approach to counteract the CFAM issue.

Demographic characteristics: Kuala Lumpur is the capital of Malaysia with a population of 1.8 million as of 2018 (13). This city is the financial and economic centre of Malaysia, attracting citizens all around the country to pursue their careers in Kuala Lumpur. Age distribution of the respondents is skewed towards younger age with a mean age of28.13 ± 10.110years due to language barrier. Older generation preferred to answer the questionnaire only if it is written in their native languages, such as Malay, Chinese and Tamil. According to the population census conducted in 2010, the percentage of Malay and Chinese population in Kuala Lumpur are 45.9% and 43.2%, respectively (14).

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Table 6 : Forward stepwise multinomial logistic regression analysis of factors associated with negative practice on CFAM

	SE	df	OR	95%CI	*p*-value
Reference: Negative practiceneutral practice vs. negative practice					
Employment status					
Employed	0.255	1	0.641	0.389:1.058	0.082
Unemployed	0.804	1	0.112	0.023:0.540	0.006*
Housewife	0.890	1	0.394	0.069:2.257	0.296
Student (control group)					
Highest level of education					
Pre-University	0.472	1	0.923	0.366:2.329	0.865
Degree	0.413	1	0.880	0.391:1.977	0.756
Postgraduate	0.560	1	3.166	1.056:9.491	0.040*
Secondary school (control group)					
Occupation					
Healthcare related	0.270	1	1.535	0.904:2.606	0.113
Non healthcare related (control group)					
Reference: Negative practicepositive practice vs. negative practice					
Employment status					
Employed	0.502	1	0.599	0.224:1.601	0.307
Unemployed	0.913	1	0.500	0.083:2.992	0.447
Housewife	1.011	1	4.181	0.576:30.329	0.157
Student					
Highest level of education					
Pre-University	1.032	1	0.336	0.044:2.540	0.290
Degree	0.727	1	1.278	0.307:5.319	0.736
Postgraduate	1.285	1	0.652	0.053:8.089	0.739
Secondary school					
Occupation					
Healthcare related	0.454	1	7.324	3.010:17.819	0.000*
Non healthcare related					
R^2 = 0.141 (Cox and Snell), 0.169 (Nagelkerke)				χ^2 (20) = 58.485	
*p<0.05					

SE: standard error, df: degree of freedom, OR: odd ratio, CI: confidence interval, x^2: chi-square

Table 7 : Correlation respondents' level of KAP on CFAM

	Knowledge	Attitude	Practice
Knowledge	1.000	0.376*	0.159*
Attitude	0.376*	1.000	0.013
Practice	0.159*	0.013	1.000

*p-value is less than 0.1

However, the percentage of Chinese respondents (61.2%) recruited in the study is higher than Malay respondents (27.1%). Malay language is the national language in Malaysia whereas English is widely used in speaking and communication. Preferenceof national language by Malay population may attribute to a low percentage of Malay participants in the study (15, 16).

***Knowledge on CFAM* :** In general, the knowledge of the general public in Kuala Lumpur on CFAM is classified as moderate (median score = 4). According to a study comparing knowledge of the population in urbanised and remote settlement, respondents from urbanised area have statistically higher knowledge than remote area. The study states that majority of respondents in urbanised area have a moderate level of knowledge, which is consistent with current finding.

The present study showed that there was a significant association between the occupation of respondent and level of knowledge on CFAM. Generally, respondents working in the healthcare-related field have better knowledge on differentiating the counterfeit medicines from their genuine counterpart. Similarly, Linus et al. reported that respondents in healthcare-related field can

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differentiate authentic antimalarial drugs from their counterfeit counterparts compared to those without healthcare-related background in Tanzania (17). A study conducted in Haryana, India revealed that more than half of the doctors working in SHKM Government Medical College (tertiary care hospital) demonstrated correct knowledge on CFAM (18). Although health care practitionersare equipped with better knowledge, platform for sharing of knowledge on CFAM with the public is not established. Gap of knowledge between health care practitioner and the public can be narrowed by educating the public on CFAM.

***Attitude towards CFAM* :** Respondents hada positive attitude towards CFAM. One of the effective ways to combat CFAM is public education about the harmful consequences of CFAM on their health (19). Although CFAM is sold at a low price, its harmful effect may incur higher health care cost to the patient in the future. Education on economic consequences of CFAM such as discouragement of the pharmaceutical company to invest in research and development of new medicine is also warranted (19).

The study has identified that the highest level of education and occupation of respondents are predictors for the level of attitude towards CFAM. Overall, health care practitioners have more positive attitude towards CFAM than of non-health care employees. The finding is consistent with a previous study conducted among Iranian pharmacists, although their knowledge and practice level on CFAM is poor, they have a positive attitude on CFAM (20). AnupNagaraj et.al also indicate that medical practitioners, dental practitioners and medical wholesaler distributors have a comparable positive attitude towards the use of CFAM in India (21).

***Practice against CFAM* :** One of the main drivers for the rapid growth of CFAM is a high demand for CFAM in the market. The willingness of consumers to purchase CFAM further aggravate the distribution of CFAM in the country (22). Good attitude of consumers towards CFAM does not necessarily result in positive purchasing practice of the consumers.

Most respondents refused to purchase the pharmaceutical product with an unknown source. The positive practice of respondents may attribute to the health care system in Malaysia. Malaysia's health care system comprised of dichotomous public and private services. Public sector provides healthcare services to cover around 65% of the population (23). Health care costs for outpatient services and admission into government hospital are covered by the budget allocated to the hospital from the central treasury (23). Malaysians are eligible for unrealistically cheap health care services with minimal payment in Malaysia. Hence, drivers of negative practice towards CFAM such as affordability and accessibility of medicines may not present in the public sector. Most health care costs of the patients admitted in private health care facilities are covered by the health insurance scheme (23). Therefore, they are less exposed to the unaffordability of medicine, which may drive them to seek cheaper alternatives for medicine.

Highest level of education, employment status and occupation were found associated with the level of practice of recruited respondents towards CFAM. Respondents with postgraduate qualification are lesslikely to have negative practice on CFAM than secondary school. Respondents with postgraduate degree capable of makingextensive decision making which requires extensive gathering and evaluation of information of products and several alternatives compared to respondents attained secondary school qualification (24). Such practices have a positive impact on purchasing intention of CFAM by respondents. Respondents are more likely to have positive practice with a comprehensive evaluation of latest information relating to the CFAM.

***Measures to combat CFAM* :** Definition of CFAM remains unclear in Malaysia. Many terms are used interchangeably with CFAM, such as fake, illegal, unregistered and substandard medicine, which may create unnecessary confusion among consumers. Different terms are used during the discussion in public health and in case of violation of intellectual property right (IPR) (19). WHO has replaced the old "substandard/spurious/falsely-labelled/falsified/counterfeit (SSFFC)" terminology with "Substandard and Falsified (SF)" due to lack of global consensus and understanding of the previous term on 2017 (25). Standardisation of the term is the fundamental approach for educating consumers and health care providers on CFAM.

Majority of the respondents agreed that education is one of several effective measures to combat CFAM. Public awareness program incorporating multifaceted approaches such as comprehensive data supported by evidence, innovative technology, communication and political commitment is effective in improving public awareness on health-related issues (26). Public must be made aware of the presence of CFAM in the market even from the legal supply chain. Imparting public with the threatening effect of CFAM can be a good focus of the

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campaign to discourage them from purchasing CFAM (27). Mass media is commonly used to influence the opinion of public towards particular issues. It may be useful to raise awareness of the public towards CFAM due to huge coverage in the country as compared to the campaign. One of the disadvantages of utilisation of mass media is short-lived of the awareness towards CFAM because issues focused by media may change over time (28). It may also decrease the confidence of the public towards conventional medicine and directs them towards alternative medicines (27).

The public is the end consumer of the pharmaceutical product. They have rights to ensure that the products are safe and quality. Lack of knowledge towards CFAM hinders them from being able to identify whether which medicines are authentic. Healthcare providers could be the main source of CFAM related information forthe general public. Nonetheless, the study respondents demonstrateda lack of confidence on the competences of healthcare providers in providing CFAM related information. This is evident that almost half of the respondents agreed that education must be provided to healthcare providers.

Limitations of this study **:** Selection and participation biases maybe present in the recruitment of participants due to the convenient sampling was used. Therefore,the sample population may not fully representative of the actual population in Kuala Lumpur. This research works well as a pilot study.It may not be possible to utilise probability sampling to recruit participants due to huge sampling frame of the population. However, location-based sampling is a feasible approach to reduce bias in the selection of participants (29).

The language barrier may also restrict the distribution of questionnaires during data collection. The questionnaire was prepared only in the English language. Although Malaysia is ranked second-best in English proficiency Index in Asia, most Malays refused to complete the questionnaire due to language preference (30). We neglected the psychological factors that influencethe willingness of public to answer the questionnaire. Therefore, a questionnaire is recommended to be prepared in three languages (English, Malay and Mandarin) for preferences of different races in Malaysia.

Since the data collection was done in Kuala Lumpur, the results may not be generalised for the entire population in Malaysia because the composition of race is different between Kuala Lumpur and Malaysia. Future studies are recommended for better assessment of KAP of public towards CFAM, taking into account the entire population from different states in Malaysia

4. Conclusion

Overall, the present study revealed that the general Malaysian public in Kuala Lumpur had a moderate level of knowledge, positive attitudes but negative practice towards CFAM. Occupation is the most important predictors for the level of KAP of respondents, followed by the highest level of education and employment status. Healthcare-related workers generally have a higher level of KAP regarding CFAM as compared to those in nonhealthcare related field. Although knowledge of respondents is positively correlated with attitude and practice on CFAM, the practice of respondents towards CFAM is unsatisfactory despite positive attitude on CFAM. Education on CFAM is recommended as one of the measures to combat the CFAM issue in Malaysia since the majority of respondents have moderately poor knowledge towards CFAM. Health care providers are encouraged to deliver information of CFAM to the public to narrow the gap of knowledge of CFAM.

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Conflict of interest

There are no financial and non-financial competing interests to be reported.

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Evaluation of Self-Medication Practice Among University Students

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Abstract

The World Health Organization refers the practice of self-medication to 'use over-the-counter medicines to treat self-diagnosed symptoms or diseases or to continue and reuse chronic medicines. Self-medication is a common and important part of the behavior of patients to cope with disease. The aim of present study is determine which classes of drugs the students used most often as self-medication. Also, to analyze the nature and views of self-medication among UCSI students, and to assess the practice of self-medication among students. A cross-sectional study was carried out for three months. A pre-validated questionnaire was distributed to the students at private University in Kuala Lumpur, Malaysia. The study was participated by 367 students. 239 (65.1%) of the respondents practiced self-medication in the past one year, among which 101(42.3%) were males and 138 (57.7%) were females. 166 (45.2%) of the respondents think self-medication is harmful. 209 (56.9%) of the respondents think that the medication used for self-medication gives symptomatic relief but does not treat the main causes of the disease. 280 (76.3%) of the respondents read the leaflet of the medication before using it. Pharmacy was the main source of self-medication. The most common indication for self-medication was fever, followed by cough, headache, common cold and pain. The most common drug classes for self-medication were antipyretics followed by cough syrups, vitamins, analgesics/anti-inflammatory and cold preparations. The three main reasons of self-medication were "health problem is not serious", "seeking quick relief" and "illness is minor". The main reasons against self-medication were "risk of using wrong medication"(80.9%), "risk of adverse effects" (64.9%)and "risk of misdiagnosis of illness"(53.4%). 279 (76.0%) of the respondents agreed that all medications including herbal have adverse effects. 354 (96.5%) was aware that increasing drug dose can be dangerous, however, only 153 (41.7%) aware that decreasing drug dose can be dangerous.The study concludes that self-medication practice was widespread among students at the UCSI university. It was indicated in conditions of mild illness such as headache, fever, cough, etc. Effective use of medications as self-medication has been of benefit to humans. Yet there are other factors that have made self-medication the main cause of drug abuse.

Key words : self-medication; university; students.

1. Introduction

Self-medication was practiced by every human being daily for their own health which was now increasingly considered as a part of self-care (1). In addition, some governments widely encouraged the practice of self-medication in the management of minor ailments (2,3). Self-medication was mostly implied when an individual encounter a common health problem that he/she thinks that a visit to doctor was not needed. Self-medication is defined as the use of any medication for self-treatment without consulting a healthcare professional (4). The term 'responsible' self-medication is frequently used in which the appropriate drugs including over the counter (OTC) drugs were indicated only when they are necessary (5). The WHO (1995) emphasized that the prevention and treatment of minor health problem can be achieved through rational self-medication at an affordable cost (6). However, this practice may cause some unwanted and serious drawbacks. There are chance of getting serious adverse outcomes, drug interactions, polypharmacy and drug abuse and dependence (6). The reasons of self-medication include high medical cost, prolonged waiting hours at clinics, time-wasting, social or family support, previous experience of similar illness and its management, lack of nearby health facilities and health professionals (3). The practice of self-medication in Malaysia was widely studied among the adult's urban population instead of students (7-9). There was only one study conducted in International Islamic University Malaysia (IIUM), Kuantan about the perceptions, knowledge, and practice of self-medication among pharmacy students (10). However, UCSI University's students may varied from the general population due to demographic variability and

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their knowledge regarding various diseases and drugs (11). The present study was carried out to evaluate the practice of self-medication among university students in UCSI University, Kuala Lumpur.

2. Materials and Methods

A cross-sectional questionnaire-based study was conducted at UCSI University, Kuala Lumpur. The target students were undergraduate and postgraduate university students. Students are recruited from 6 different faculties. The sample size was assumed to have a confidence level of 95%, 5% margin of error, 50% recruitment rate and a maximal sample size of 8000 students. According to the Rao soft sample size calculator (http://www.raosoft.com/samplesize.html), the minimum required sample size, was 367 respondents.

The study subjects were informed that participation will be completely voluntary, and the information collected would be anonymous. The questionnaire was printed in English language and consists of four parts which was modified from the other studies (3,4,11,12). The preliminary letter explained the term 'self-medication' and require the participants to report on the use of self-medication during past one year. Part 1 focused on the practice of self-medication in the past one year. Part 2 was used to assess the common drugs and indications for self-medication. Part 3 was used to examine the factors affecting the practice of self-medication. Part 4 evaluated the students' view on some aspects of self-medication.

The data collected was analyzed using SPSS version 20. The descriptive data and categorical variables were expressed as counts/frequency and percentages. In addition, continuous variables were summarized as mean (standard deviation). Independent sample t-test was utilized to compare between male and female groups where $p<0.05$ was considered statistically significant.

3. Results and Discussion

Only 367 out of 381 total students completely filled the survey with response rate of 96.3%. Incomplete filling of the questionnaires and the questionnaires with unsigned consent forms were not included in this study. In addition, some of the respondents did not submit their questionnaires for data analysis. Among 367 respondents, 149 (40.6%) were males, whereas 218 (59.4%) were females. The mean age ± standard deviation was 20.88 ± 1.81 years. This study recruited 142(38.7%) first-year undergraduate students; 81(22.1%) second-year undergraduate students; 95(25.9%) third-year undergraduate students; 38 (10.4%) fourth-year undergraduate students and 11(3.0%) postgraduate students. Among 367 respondents, 94 (25.6%) were medical students, whereas, 273 (74.4%) were from non-medical courses. The characteristics of participating students are described in Table 1.

Table 1. Demographic characteristics of respondents (N=367)

Age (Mean ± SD)	20.88 ± 1.81	
Gender	**Frequency**	**Percentages (%)**
Male	149	40.6
Female	218	59.4
Faculty		
a) Medical		
Faculty of Pharmaceutical Sciences	79	21.5
Faculty of Medicine & Health Sciences	15	4.1
Total	94	25.6
b) Non-medical		
Faculty of Business & Information Sciences	116	31.6
Faculty of Engineering, Technology & Built Environment	87	23.7
Faculty of Applied Sciences	44	12.0
Faculty of Social Sciences & Liberal Arts	26	7.1
Total	273	74.4
Level of study		
First year undergraduate	142	38.7
Second year undergraduate	81	22.1
Third year undergraduate	95	25.9
Fourth year undergraduate	38	10.4
Postgraduate	11	3.0

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Self-medication practice by the students in the past one year

Self-medication was practiced by 239 (65.1%) of the respondents in the past one year, among which 101 (42.3%) were males and 138 (57.7%) were females. 166 (45.2%) of the respondents think self-medication is harmful. Majority (91.8%) of the respondents practice self-medication not less than once in the past year. 244 (66.5%) of the respondents had an idea about rational drug use. 209 (56.9%) of the respondents think that the medication used for self-medication gives symptomatic relief but does not treat the main causes of the disease. 247 (67.3%) of the respondents realized about the potential adverse effects of self-medicated drugs. 280 (76.3%) of the respondents read the leaflet of the medication prior consumption. According to Table 2, majority (91.8%) of the respondents practiced self-medication at least once in the past year, which was contradictory to the first question in Table 2 (65.1%).

The pharmacy was the main source of self-medication for 273 (74.4%) of the respondents followed by home stock (16.3%), herbal store (7.1%), street market (1.1%) and friends (1.1%). 214 (58.3%) of the respondents speak to a pharmacist before taking a drug as self-medication. 100 (27.2%) of the respondents had been consumed antibiotics as a part of self-medication during the past one year. More females than male respondents were having an idea about rational drug use and read the leaflet of the medicine before consumption (p=0.041, 0.030) as shown in Table 2.

Table 2. Shown self-medication practice by the students in the past one year.

Did you have any self-medication in the past one year?	Male (%)	Female (%)	Total (%)	p (t-test)	
Yes	101(67.8)	138 (63.3)	23965.1)		0.378
Have you ever treated yourself with medication without it to be prescribed by a doctor?					
Yes	116(77.9)	172 (78.9)	288 78.5)		0.811
Do you think self-medication is harmful?					
Yes	65 (43.6)	101 (46.3)	166 45.2)		0.610
How many times have you practiced self-medication and used over the counter drugs in the past year?					
⌟ No	12 (8.1)	18 (8.3)	30 (8.2)		0.740
⌟ 1 Time	39 (26.2)	49 (22.5)	88 (24.0)		
⌟ 2 Times	39 (26.2)	60 (27.5)	99 (27.0)		
⌟ 3 Times	21 (14.1)	37 (17.0)	58 (15.8)		
⌟ 4 Times	6 (4.0)	5 (2.3)	11 (3.0)		
⌟ 5 Times	3 (2.0)	4 (1.8)	7 (1.9)		
⌟ >5 Times	29 (19.5)	45 (20.6)	74 (20.2)		
How long was the average duration of self-medication?					
⌟ No	5 (3.4)	9 (4.1)	14 (3.8)		0.869
⌟ 1-2 days	73 (49.0)	99 (45.4)	172(46.9)		
⌟ 2-3 days	28 (18.8)	57 (26.1)	85 (23.2)		
⌟ 3-4 days	19 (12.8)	21 (9.6)	40 (10.9)		
⌟ 4-5 days	7 (4.7)	5 (2.3)	12 (3.3)		
⌟ 5-6 days	1 (0.7)	2 (0.9)	3 (0.8)		
⌟ 1 week	5 (3.4)	11 (5.0)	16 (4.4)		
⌟ More than a week	11 (7.4)	14 (6.4)	25 (6.8)		
Do you have any idea about rational drug use?					
Yes	90 (60.4)	154 (70.6)	244(66.5)		**0.041**
Do you think that the medication you use to treat yourself gives symptomatic relief but does not treat the main causes of the disease?					
Yes	76 (51.0)	133 (61.0)	209(56.9)		0.058
Did you know the potential adverse effects of the drug by which you self-medicated?					
Yes	94 (63.1)	153 (70.2)	247(67.3)		0.156
When you treat yourself with a medication, do you read the leaflet of the medication before using it?					
Yes	105(70.5)	175 (80.3)	280(76.3)		**0.030**
What sources of self-medication do you use?					
⌟ Pharmacy	110(73.8)	163 (74.8)	273(74.4)		0.706
⌟ Home stock	24 (16.1)	36 (16.5)	60 (16.3)		
⌟ Street market	2 (1.3)	2 (0.9)	4 (1.1)		
⌟ Herbal store	11 (7.4)	15 (6.9)	26 (7.1)		
⌟ Friends	2 (1.3)	2 (0.9)	4 (1.1)		
Do you speak to pharmacist before taking a drug as self-medication?					
Yes	87 (58.4)	127 (58.3)	214(58.3)		0.980
Have you ever taken antibiotic as self-medication?					
Yes	45 (30.2)	55 (25.2)	100 27.2)		0.295

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Indications and drug classes for self-medication

The most common health condition for self-medication was fever (72.8%), cough (67.6%), headache (67.0%), common cold (65.7%) and pain (30.5%). Other indications include diarrhea (28.6%), mouth ulcers (26.4%), gastric pain (22.6%), allergy (15.8%), constipation (9.5%), fungal/microbial infection (4.9%), insomnia (3.8%), sex-related problem (2.2%), contraception (1.6%) and the least was sore throat (0.5%). The use of self-medication for headache was more common in males than females (78.5% vs. 59.2%, p=0.000).

The most common drug classes for self-medication was antipyretics (59.7%), cough syrups (59.1%), vitamins (55.3%), analgesics/anti-inflammatory (45.8%) and cold preparations (36.0%). Other than that, nutritional supplements (34.3%), nasal/ear/eye drops (34.1%), herbs (21.8%), anti-gastritis (21.3%), antihistamines (19.6%), topical agents (17.4%) and anti-diarrhea (17.2%) were some other examples of drug classes indicated for self-medication. Nasal/ear/eye drops, topical agents and oral contraceptives were more commonly used by females than males (p=0.002, 0.025, 0.027).

Table3. Shown indications and drug classes for self-medication.

Indications	Male (%)	Female (%)	Total (%)	p (t-test)
Fever	114(76.5)	153(70.2)	267(72.8)	0.182
Cough	106(71.1)	142(65.1)	248(67.6)	0.229
Headache	117(78.5)	129(59.2)	246(67.0)	**0.000**
Common cold	96(64.4)	145(66.5)	241(65.7)	0.681
Pain	46(30.9)	66(30.3)	112(30.5)	0.903
Diarrhea	46(30.9)	59(27.1)	105(28.6)	0.429
Mouth ulcers	41(27.5)	56(25.7)	97(26.4)	0.697
Gastric pain	32(21.5)	51(23.4)	83(22.6)	0.667
Allergy	19(12.8)	39(17.9)	58(15.8)	0.186
Constipation	16(10.7)	19(8.7)	35(9.5)	0.518
Fungal/Microbial infection	6(4.0)	12(5.5)	18(4.9)	0.521
Insomnia	3(2.0)	11(5.0)	14(3.8)	0.137
Sex-related problem	4(2.7)	4(1.8)	8(2.2)	0.585
Contraception	2(1.3)	4(1.8)	6(1.6)	0.716
Sore throat	1(0.7)	1(0.5)	2(0.5)	0.787

Factors affecting the practice of self-medication

The three main causes of self-medication were "health problem is not serious" (65.1%), "seeking quick relief" (62.1%), "illness is minor" (53.7%) and the least was "I do not trust my physician" (0.8%). Among all the reasons, avoidance of long waiting at clinics, suggestion of a relative/friend and embarrassed of discussing own symptoms were more common in males than females in order to acquire self-medication ($p<0.05$). More females go for self-medication due to "illness is minor" than males ($p = 0.006$).

The three main reasons for seeking professional help were "symptoms are worsening" (71.1%), "symptoms last for more than one week" (64.9%), "thinking the problem is serious" (64.6%) and the least was "in case of the mental problem" (6.8%). The reasons for seeking professional help was not statistically significant difference between male and female group ($p > 0.05$).

The major causes against self-medication were "risk of using wrong medication" (80.9%), "risk of adverse effects" (64.9%), "risk of misdiagnosis of illness" (53.4%), "risk of drug interaction" (35.7%) and "risk of drug abuse and dependence" (32.2%). The risk of misdiagnosis of illness was the most commonly reported reason against self-medication in females than males (p=0.041) as shown in Table 4.

Student's views on some aspects of self-medication

279 (76.0%) of the respondents agreed that all drugs including herbal cause adverse effects. 354 (96.5%) of the respondents were aware of the danger of increasing drug dose, however, only 153 (41.7%) aware of the danger of decreasing drug dose. Most of the respondents were aware that concurrent use of drugs can be dangerous (94.0%), physician help must be sought in case of adverse effects (95.9%) and using medications with unknown substances in patients with liver and kidney disease is

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Table 4. Shown factors affecting the practice of self-medication. p< 0.05 was considered significant when compared between male and female groups (N=367)

Reasons in favor of self-medication	Male (%)	Female (%)	Total (%)	p (t-test)
Health problem is not serious	89(59.7)	150(68.8)	239(65.1)	0.074
Seeking quick relief	100(67.1)	128(58.7)	228(62.1)	0.104
Illness is minor	67(45.0)	130(59.6)	197(53.7)	**0.006**
High cost of medical consultation	72(48.3)	86(39.4)	158(43.1)	0.092
Avoidance of long waiting at clinics	46(30.9)	43(19.7)	89(24.3)	**0.014**
Suggestion of a relative/friend	25(16.8)	21(9.6)	46(12.5)	**0.042**
Physician's advice of self-management	13(8.7)	9(4.1)	22(6.0)	0.069
Embarrassed of discussing own symptoms	8(5.4)	1(0.5)	9(2.5)	**0.003**
I do not trust my physician	2(1.3)	1(0.5)	3(0.8)	0.357
Reasons for seeking professional help				
Symptoms are worsening	109(73.2)	152(69.7)	261(71.1)	0.478
Symptoms last for more than one week	101(67.8)	137(62.8)	238(64.9)	0.332
Thinking the problem is serious	95(63.8)	142(65.1)	237(64.6)	0.787
Presence of severe pain	97(65.1)	131(60.1)	228(62.1)	0.333
Usual treatment is not effective	76(51.0)	109(50.0)	185(50.4)	0.850
Side effects of usual treatment	21(14.1)	23(10.6)	44(12.0)	0.306
In case of mental problem	10(6.7)	15(6.9)	25(6.8)	0.950
Reasons against self-medication				
Risk of using wrong medication	123(82.6)	174(79.8)	297(80.9)	0.514
Risk of adverse effects	96(64.4)	142(65.1)	238(64.9)	0.889
Risk of misdiagnosis of illness	70(47.0)	126(57.8)	196(53.4)	**0.041**
Risk of drug interaction	45(30.2)	86(39.4)	131(35.7)	0.070
Risk of drug abuse and dependence	49(32.9)	69(31.7)	118(32.2)	0.804

dangerous (95.6%). 195 (53.1%) of the respondents agreed that no drugs can be used during pregnancy. 62.9% approved that mild illnesses do not require drug treatment whereas 69.2% approved that self-medication can mask clinical presentations of the illness so that the physician can overlook them easily as shown in Table 5.

More females than males were aware of the aspects of self-medication that "physician help must be sought in case of adverse effects" (p=0.036) and "mild medical problems do not require drug treatment" (p=0.025) as shown in Table 6.

Table 5. Shown student's views on some aspects of self-medication (N=367)

Student's views on self-medication	Approve (%)	Disapprove (%)
All medications (prescription, OTC and herbal) have adverse effects.	279(76.0)	88(24.0)
Concomitant use of drugs can be dangerous.	345(94.0)	22(6.0)
Increasing drug dose can be dangerous.	354(96.5)	13(3.5)
Decreasing drug dose can be dangerous.	153(41.7)	214(58.3)
Physician help must be sought in case of adverse effects.	352(95.9)	15(4.1)
Using medications with unknown substances in patients with liver and kidney disease is dangerous.	351(95.6)	16(4.4)
No drug can be used during pregnancy.	195(53.1)	172(46.9)
Mild medical problems do not require drug treatment.	231(62.9)	136(37.1)
Self-medication can mask signs and symptoms of disease so the physician can overlook them easily.	254(69.2)	113(30.8)

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Table 6. Shown comparison of student's views on self-medication between male and female group (N=367)

Student's views on self-medication	Approve (%)		Disapprove (%)		p value (t-test)
	Male	Female	Male	Female	
All medications (prescription, OTC and herbal) have adverse effects.	115(77.2)	164(75.2)	34(22.8)	54(24.8)	0.668
Concomitant use of drugs can be dangerous.	140(94.0)	205(94.0)	9(6.0)	13(6.0)	0.976
Increasing drug dose can be dangerous.	143(96.0)	211(96.8)	6(4.0)	7(3.2)	0.679
Decreasing drug dose can be dangerous.	61(41.0)	92(42.2)	88(59.1)	126(57.8)	0.810
Physician help must be soughtin case of adverse effects.	139(93.3)	213(97.7)	10(6.7)	5(2.3)	**0.036**
Using medications with unknown substances in patients with liver and kidney disease is dangerous.	143(96.0)	208(95.4)	6(4.0)	10(4.6)	0.797
No drug can be used during pregnancy.	83(55.7)	112(51.4)	66(44.3)	106(48.6)	0.416
Mild medical problems do not require drug treatment.	104(69.8)	127(58.3)	45(30.2)	91(41.7)	**0.025**
Self-medication can mask signs and symptoms of disease so the physician can overlook them easily.	102(68.5)	152(69.7)	47(31.5)	66(30.3)	0.797

The study showed that UCSI university students commonly practiced self-medication for mild medical illnesses to achieve quick relief. This cross-sectional survey has shown that the mean age of the respondents was similar to the previous studies conducted in Egypt and Jordan reported (11,13).

The response rate of the current study (96.3%) was higher than the study conducted in the United Arab Emirates and Jordan (6,11). This may be due to the method of data collection by using both web-based and paper-based questionnaires in this study. In addition, the respondents in this study were highly cooperative and well understand the purpose of the study conducted. In contrast, the study conducted in the United Arab Emirates and Jordan utilized paper-based questionnaires only (6,11).

This study has shown that the university students practiced self-medication similarly to the study conducted in Egypt and Turkey (13,15). In contrast, the prevalence of self-medication in Slovenia (92.3%) was greater than the current study (4). This may be due to the larger sample size of 1294 students in Slovenia compared to our study (4). Furthermore, the practice of self-medication was greater in Iran, Bangladesh, Jordan, United Arab Emirates, and Ethiopia (11,12,14,16,17). This may be due to the involvement of pharmacy or health science students only in the studies. Health science students are familiar with the signs and symptoms of diseases in order to practice self-medication and are well equipped the knowledge of self-medication in mild medical problems. Apart from that, the study conducted in Karachi and Nigeria had a higher prevalence of self-care than our study (18,19). This may be due to the higher number of healthcare students, family education and the easy availability of the drugs. It was clear that the pattern of self-medication varies among the countries because of geographical, demographic, and economical variation.

In this current study, there was no significant difference in the practice of self-medication among genders, similarly with the study conducted in Jordan, Iran and Bangladesh (11,16,17). However, the study conducted in Egypt and Nigeria reported that self-medication was significantly associated with gender. This may due to the unequal distribution of male and female respondents in the study conducted in Egypt and Nigeria (13,19).

Based on the current study, more than half of the university students were aware of rational drug use consistent with the study conducted in the United Arab Emirates and Turkey (12,15). However, in Jordan, a

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higher percentage of the students were aware of rational drug use than our study, this may be due to the inclusion of pharmacy students only in their study (11). Awareness towards rational drug use is vital to reduce healthcare burden, the occurrence of adverse effects and the chance of antibiotics resistance (20).

In addition, the current study reported that university students think that the medication used to treat them give symptomatic relief but does not treat the main causes of the disease. Similarly, in Jordan, the pharmacy students think that the medication used to treat them gives symptomatic relief but does not treat the main cause of the disease (11).

In this study, majority of the respondents read the leaflet of the medication before using it which was consistent with the study conducted in Bangladesh, Egypt and Jordan (11,13,17). The habit of reading medication's leaflet is important to avoid the misuse of self-medicated drugs.

According to the current study, pharmacy was the main source of self-medication, similarly in Jordan, Egypt and the United Arab Emirates (11-13). This is because pharmacies are easily available and provide a wide range of over the counter (OTC) medication and prescription-only drugs.

Moreover, 27.2% of the university students in this study had taken antibiotics as self-medication. Similarly, 36.9% of university students in Turkey and 32.0% of university students in the United Arab Emirates had taken antibiotics as self-medication (12,15). In contrast with Nigeria, only 10.5% of the university students had taken antibiotics as self-medication (19). The relatively lower percentage of antibiotic use in Nigeria than our study may be due to the high cost of antibiotics and the high awareness of the students towards the threat of antibiotics resistance (20,21). Hence, pharmacist plays a crucial role in dispensing antibiotics only if the prescription is presented. In our study, there was no significant difference in the use of antibiotics as self-medication between genders, similarly in Turkey (15). However, in Jordan, more males had taken antibiotics as self-medication compared with females (11).

The main indications for self-medication among university students in this study were fever, cough, headache, common cold, pain, diarrhea, mouth ulcers, gastric pain, allergy, constipation and fungal/microbial infection, similarly in Bangladesh (17). The outcomes of other studies conducted in Egypt, Jordan, Ethiopia and Iran were inconsistent with our findings (11,13,14,16). The differences among the studies are attributed to the demographic variation, lifestyles, diet consumption, education and sexual behavior of the university students involved in the studies.

In the current study, more males than females had been self-medicated for headaches (p=0.000). However, studies showed that headache was more prevalent in females than males due to hormonal differences especially during menstruation and less tolerance towards the sensation of pain (22). Hence, the difference between this study and the other studies may be due to the higher number of male respondents that cause bias in the result.

According to this study, the most common drug classes for self-medication were antipyretics, cough syrups, vitamins,analgesics/anti-inflammatory, cold preparations, nutritional supplements, nasal/ear/eye drops and herbs. In contrast with the United Arab Emirates, the most common drug classes were analgesics, antipyretics, vitamins and minerals and herbal teas. The more frequent use of vitamins and herbs in the United Arab Emirates than our study may be due to the knowledge of students towards the use of vitamins and the cultural beliefs of the students. Generally, the use of vitamins and minerals are becoming more popular among university students that are widely exposed to the health-related information (23). In addition, the students in the United Arab Emirates are more knowledgeable about the use of traditional medicine and the perception that the use of herbals was cheap, effective and without side effects. However, a recent study concluded that the use of herbs may cause liver toxicity and kidney toxicity due to overdose, drug-drug interaction and the lack of control from the Drug Control Agency (22). On the other hand, our study was inconsistent with the study conducted in Bangladesh and Iran (16,17).

According to this study, the fundamental cause of self-medication were health problem is not serious, seeking quick relief, illness is minor, high cost of medical consultation and avoidance of long waiting at clinics, while the least was "I do not trust my physician" similarly with the study carried out in the United Arab Emirates (12). However, in Karachi, the major reasons were the problem is not serious, previous experience, lack of time, cost of the consultation, the urgency of the problem, advice from friends and unavailability of transport (18). Also, a study conducted in Egypt reported that minor disease, knowledge from previous experience, same drugs prescribed by a doctor, save money and time, fast relief and the least was the unavailability of health service were the main causes of self-medication (13). In Iran, the main causes of self-medication among health science students were previous experience about the similar illness, mild disease, drug availability and history of drug use (16).

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Furthermore, a study conducted in Jordan reported that the main reasons of self-care were time saving, health problem was not serious and ease of drug availability (11). In Bangladesh, the main reasons for self-medication were illness is minor, easy availability of medicine and emergency (17).

In this study, the two major reasons for seeking professional help were symptoms are worsening (71.1%) and symptoms last for more than one week (64.9%) similarly in Slovenia (4). In contrast with the studies in Jordan, and the United Arab Emirates. In addition, our study concluded that the reasons for seeking medical advice were not statistically significant between genders, whereas, the other studies did not investigate the relationship between the reasons for seeking professional help and genders (4,11-19).

Based on our study, the reasons against self-medication were the risk of using the wrong medication, risk of adverse effects, risk of misdiagnosis of illness, risk of drug interaction and risk of drug abuse and dependence, consistent with United Arab Emirates (12). According to our study, more females than males assumed that the risk of misdiagnosis of illness was the reason against self-medication ($p= 0.041$) while the other reasons were not significantly different between genders. On the other hand, the other studies did not investigate the significant difference between the reasons against self-medication and genders (4,11-19).

According to this study, majority of the university students approved that "increasing drug dose can be dangerous" similarly in United Arab Emirates, Jordan and Bangladesh (11,12,17). Besides that, our study showed that majority of the students were aware that "physician help must be sought in case of adverse effects" similarly in Jordan and Bangladesh (11,17). In our study, most of the students agreed that "using medications with unknown substances in patients with liver and kidney disease is dangerous" and "the concomitant use of drugs can be dangerous", consistent with a study conducted in Jordan (11). However, our findings showed that more students (76.0%) were aware that "all medications (prescription, OTC and herbal) have adverse effects" in contrast with United Arab Emirates (41%) and Jordan (49%) (11,12). Besides that, our study reported that more than half of the respondents disagreed on the danger of decreasing drug dose can be dangerous, while 30-60% of the students disagreed that "no drug can be used during pregnancy", "mild medical problems do not require drug treatment" and "self-medication can mask signs and symptoms of disease so the physician can overlook them easily", similarly in the United Arab Emirates and Bangladesh (12,17). Hence, university students should be educated on the effects of drug dose, drug use during pregnancy, the rationale of appropriate drug treatment in the maintenance of good health. In our study, more females than males approved that "physician help must be sought in case of adverse effects" ($p=0.036$) and "mild medical problems do not require drug treatment" ($p=0.025$). However, the other studies did not examine the significant differences between student's perceptions on self-medication and genders (4,11-19). The limitations of this current study were attributed to the evaluation of self-medication practice in the past year that may cause recall bias among respondents. This is because the respondents tend to forget their previous use of drugs as self-care in the past one year. In addition, self-reported basis of the questionnaires may cause over-reporting or under-reporting of the responses. The inclusion of more centers and larger sample size would enable us to better characterize the practice and perception of self-medication among students.

4. Conclusion

Self-medication was commonly practiced among university students. The most common indications for self-medication among university students were fever, cough, headache, common cold, pain, diarrhea and etc. The knowledge of students regarding the reasons for and against self-medication seems appropriate. The current study recommends that proper education should be done among university students to prevent and treat some common indications for self-medication by incorporating health-related knowledge as a part of the curricula in all university programs.

Ethical approval

The study has received the ethics initial approval: NMRR-19-1469-48803 (IIR), Reference: KKM/ NIHSEC/P19-1406(6), Date: 13-August-2019.

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Conflict of interest

The authors declare no conflict of interest.

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Course Satisfaction and Perception of Malaysian Provisionally Registered Pharmacists Towards their Training : A Qualitative Study

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Abstract

In Malaysia, it is compulsory for pharmacy graduates to undergo a one year provisionally registered pharmacist (PRP) training. The liberalisation of PRP training following the saturation of governmental institutions has raised many concerns about the transformation of the training into a scheme for exploiting the fresh pharmacy graduates without a systematic training as its initial purpose. The objective of this study is to explore the experiences and perceptions of Malaysian pharmacists about PRP training at different settings, after the liberalisation of PRP training.A qualitative study was conducted in West Malaysia, mainly in Klang Valley. Through maximum variation purposeful sampling, data were gathered from 33 participants from different settings included government hospital, private hospital, health clinic, community pharmacy, manufacturing pharmaceutical industry, non-manufacturing pharmaceutical industry and research and development (academia) using semi-structured in-depth interview method. All the interviews were audio-recorded and conducted in English. The data were analyzed according to framework approach.A total of 4 themes and 24 codes were identified in this study, the themes included placement, payment, working condition and training. The findings indicated a balance of positive and negative perceptions towards the PRP training in various settings. The participated pharmacists believed that PRP training was a necessary exposure to gain required experiences, despite all difficulties and challenges. The focus of government in the past few years was to resolve the saturation of PRP placement in government hospitals through the liberalisation of PRP training. Emphasis should be in improving the inconsistency of quality in PRP training program in different practice settings in order to improve the experiences and perceptions of future PRPs in their training.

Key words : Provisionally Registered Pharmacist (PRP); Pre-registration Training; Malaysia; Pharmacy; Pharmacist

1. Introduction

In Malaysia, pharmacists are required to have successfully completed an undergraduate pharmacy degree program for a minimum of 4 years duration from an accredited public or private higher learning institution (1) and passed the qualifying examination for practicing as a pharmacist (2), prior to enrolling in a one year compulsory training. The compulsory training known as provisionally registered pharmacist (PRP) training can be done in any training premises approved by Pharmacy Board of Malaysia (PBM), either in public or private sector such as government hospital, public institution, health clinic, community pharmacy, private hospital, research and development (academia), manufacturing and non-manufacturing pharmaceutical industry (3). The aim of the PRP training is to provide pharmacists with sufficient in-depth clarity in the understanding of pharmacy practice and to equip the pharmacists with relevant knowledge and skills by exposing them to the real world setting through hands-on training modules (4-6).

The upsurge in the number of pharmacy graduates in Malaysia in the past few years has been driven in part by the expansion of higher learning institutions worldwide and nationwide (7). Before 1996, only one public institution offered pharmacy degree program (7-8), presently there are 19 institutions in Malaysia and 71 institutions from other countries offering pharmacy degree program that recognized by PBM (1). The rate of pharmacy graduates has been increased up to 1,400 annually (9-10). These rising numbers have recently caused a point of saturation in the PRP placement for pharmacy graduates to undergo PRP training (10). The liberalisation of PRP training into the private sectors, as the alternative training premises to government hospitals, has been introduced by the PBM since October 2012. The

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primary aim of liberalisation is to resolve the saturation of PRP placement in government hospitals and in furtherance to provide more training opportunities for the pharmacy graduates (10-12). In 2017, the Ministry of Health has introduced a new policy to offer a one year service contract to pharmacy graduates, with a maximum contract of two years. This is to reduce the waiting time for the training placement at government sector due to the constraints in permanent posts (13-14).

Previous studies have investigated the perception of PRP towards their training at government hospitals in Malaysia (4, 15, 16). These studies pointed out the positive and negative perceptions of the pharmacists toward PRP training, however suffer from major drawbacks in terms of scope, sample size and methodology (4, 15, 16). The lack of reliable research on the liberalisation of PRP training following the saturation of governmental institutions has raised many concerns about the transformation of the training into a scheme for exploiting the fresh pharmacy graduates without a systematic training as its initial purpose, requisite an in-depth qualitative investigation.The primary objective of this study is to explore the pharmacists' experiences and perceptions about PRP training at different settings. This study also provides an opportunity to improve the current PRP training program based on the suggestions from interviewees.

2. Materials and Methods

Study design

The present qualitative study was conducted using in-depth interview method to develop a comprehensive understanding of the topic (17).

Recruitment of participants

All Malaysian pharmacists who had completed PRP within the past 2 years or those who were currently doing PRP for at least 6 months had been eligible to participate in this study. Maximum variation purposeful sampling was performed to make sure that they were the representatives of different practices settings in this study. Selected pharmacists were invited through social media, email and personal connections. The recruitment of participants began in June until August 2018. A total of 33 participants from different PRP training sectors were participated in the study. There were 5 participants from each sector except manufacturing pharmaceutical industry and research and development that had 4 participants each. One of the participants underwent PRP training in community pharmacy for 11 months and later changed to government hospital setting. The data from both settings was collected.

Data collection

The data collection started from June to September 2018. A semi-structured face to face interview was carried out with prior arrangement with the participants at a place and time of their convenience. The place of interview was mainly in Klang Valley (an area comprises of Kuala Lumpur and conjoining cities and towns of Selangor state) (18), except two interviews conducted at Melaka and one interview conducted at Ipoh. The verbal informed consent was obtained from all participants before the interview began and the study's objectives were stated and explained to them. A pre-determined set of open-ended questions were asked during the interview and each interview lasted from 30 minutes to 50 minutes. All the interviews were audio-recorded using a voice recorder and conducted in English. The interview was continued until no new data, themes and coding were captured in the interviews (19).

Data analysis

As a preliminary step, the plausible themes and codes were developed, based on already-agreed-on professional definitions found in literature reviews; from local and commonsense constructs; from researchers' values, theoretical orientations and personal experiences. The data analysis started with familiarizing with the data at the same time as the interviews were being transcribed verbatim. The audio-recorded files were listened repetitively and the transcriptions were reread several times to provide leads for further data gathering and provoke insights. Once all of the interviews had been transcribed and checked, the transcriptions were examined line by line to assign codes and sub codes that denote particular meaningful segments as used in a grounded theory approach (20). This coding combines both deductive and inductive approaches. The sub codes were compared in terms of similarities and differences, and those that implied the same meaning were assigned to one code which were further grouped into themes that reflected their central content. New sub codes were placed in previous code after assessment and code were formed as the data analysis continued. To further improve the clarity and reduce the ambiguity of the framework, sub codes were compared and if possible merged, relabelled, split as necessary and placed in a common code. By the end of this stage, all of the themes were generated to indicate the general content of the codes and sub codes. Upon completion of analysis, data saturation was attained

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at 21st interviews. Additional interviews were conducted to ensure no new or relevant information were emerging. Subsequently, the data was tabulated into a thematic framework (Table 1). A total of 4 themes and 24 codes were identified. At last the final conclusion was drawn from the rich data.

Ethical consideration

This study was approved by the Medical Research and Ethics Committee, Ministry of Health Malaysia (NMRR-18-2009-42980) and Faculty Research and Scholar Activities (FRSA), Faculty of Pharmaceutical Science, UCSI University.Confidentiality and anonymity

Table 1 : Thematic Framework

Themes	**Codes**	**Total number of respondent (n=34)**	**Consideration about PRP pharmacist**
Placement	Waiting time	32	All settings
	Selecting the PRP training centre	34	All settings
	Emotional status of graduates during PRP waiting period	31	All settings
	Pros of PRP training centre	34	All settings
	Cons of PRP training centre	34	All settings
Payment	Agreement on allowance	34	All settings
	Minimum allowance	34	All settings
	Supplementary allowances	34	All settings
Working condition	Status of PRP	34	All settings
	Comparison between PRP and employee	34	All settings
	Working hour	34	All settings
	Leave	33	All settings
	Workload	34	All settings
	Comparison of workload of PRP and registered pharmacist	34	All settings
	Comparison of responsibility between PRP and registered pharmacist	27	G, P, H, C, M, NM
	PRP training in line with pharmacist's job scope	34	All settings
	Accessibility to computerized system	23	G, P, H, M, NM
Training	Regular and systematic training for PRP	34	All settings
	Difference between PRP training and academic attachment	34	All settings
	Sufficiency of undergraduate program	34	All settings
	Incorporation of PRP training into university curriculum	32	All settings
	Preceptor's qualification	34	All settings
	Relationship with preceptor	33	All settings
	Experiences of PRP	34	All settings

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were informed and assured to all participants during the invitation stage that any type of disseminating of the data will be done anonymously and both PRP training centre and pharmacist will be unidentified. The verbal informed consent was obtained and audio recorded from all the participants at the beginning of the interview.

3. Results and Discussion

Characteristics of participants

Out of 33 participants, 23 of them were PRP and the remaining 10 participants were FRP. Most of the participants (n =19) from government hospital, private hospital, health clinic and research and development were female. In pharmaceutical industry, most of the participants were male. Majority of participants (n = 20) stayed near to the workplace and travelled by car (n = 26).

Main themes

The representative quote for each group of responses is shown to illustrate themes and codes.

Placement

Waiting time

Most of the participants waited less than 6 months for the PRP placement from their graduation. During the waiting period, they were working as a pharmacist assistant or intern to gain experiences in the relevant pharmacy field or went for vacation. Participants who waited more than 6 months for the PRP placements were those who applied for PRP training in government hospital. Those who failed to obtain a placement in government hospital,opted for private hospital or health clinic to do their PRP training. The acceptable waiting period for majority for PRP placement was 6 months.

Around 5 to 6 months. I feel compared to senior is not a very long duration. 5 to 6 months is enough for me to have some rest at the same time to work part-time to gain some experiences before going to government hospital.

(Government hospital, Interview 4, Line 11)

Cons of PRP training centre

The main short coming of the PRP training centre was limited learning experience. Participants in private hospital had limited exposure on clinical, TDM and TPN due to the short training duration or unavailability of the services. Participants under health clinic, non-manufacturing pharmaceutical industry and research and development reported that the PRP training program had not been well structured and organized. This was probably due to the newly established of PRP training module in the individual premise with poor guidance which affected their learning experience.

Limitation is because we are still in new module so sometimes our preceptor don't know how to handle us they don't know what should we do for in order to fulfil the logbook all that. For example, for unit inspection normally our pharmacist they will go outside for unit inspection but for PRP she doesn't know how to conduct for the unit inspection but we just follow the pharmacist to go for unit inspection.

(Health clinic, Interview 4, Line 42, 46)

Payment

Minimum allowance

The payment of PRPs were in line with the minimum allowance policy except those under research and development setting. Some received monthly research grant lower than the minimum allowance and some were not receiving any allowances or research grant throughout the training. Participants from government sector had the same basic allowance, while for other settings, allowance varied from one training premises to another.

I actually being paid by the grant, there is this fix amount that everybody get. The grant is for the research project, my supervisor will allocate some for the computer, some for the software, some for my allowance. Actually very low because we are still student and I employed here as research assistant.

(Research and development (academia), Interview 2, Line 64, 66, 69)

Working condition

Work load

The workload of PRPs in government hospitals was different from one department to another. Outpatient pharmacy had the highest workload due to high volume of patients and large amount of prescriptions. Overall, their workload was reported to be manageable, likewise in health clinic setting. In private hospitals, their workload was mostly operation and pharmacy management. Heavy workload was reported in manufacturing sector as some of them were placed in a specific department for an extended period and they were required to handle multiple work tasks concurrently. The workload of PRP in non-manufacturing pharmaceutical industry was depend on the projects available at that time. Only selected work

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tasks can be delegated to PRPs because one project required a few months to be completed which consequently limit their job scope and learning experience. In community pharmacy, the amount of workload was determined by the PRPs' job scope. They have more workload if they are involved in branch opening, warehouse sale, management work and others. For research and development, the 9 months research training was heavy but manageable and the 3 months hospital training was hectic due to the high requirements of logbook.

Workload for PRP definitely very tough because besides operation work we do have a lot of management work to do and have to fill in the logbook and spend extra time to study.

(Private hospital, Interview 1, Line 203)

PRP training in line with pharmacist's job scope

Except the participants from pharmaceutical industry, all other participants agreed that the PRP training was in line with pharmacist's job scope. In manufacturing pharmaceutical industry, they were involved in technical, management and administrative work in addition to pharmacist related work. Likewise, in non-manufacturing sector, their job scope was wide-ranging across departments, for instance regulatory affair and pharmacovigilance was more towards a typical pharmacist's role compared to sales and marketing department.

I'm not too sure as a pharmacist, but it is not the same as a clinical pharmacist. If deal with the regulatory side, yes, will be in line with a pharmacist, if sale and marketing it is more to selling, promoting and marketing the products, so I wouldn't say is in line but we can use some pharmacy knowledge in sale and marketing.

(Non-manufacturing pharmaceutical industry, Interview 1, Line 118)

Training

Regular and systematic training for PRP

A vast majority of participants were uncertain whether regular and systematic PRP training was provided to them, especially those under private sector. Half of them agreed the PRP training was regular and systematic. Due to the nature of each training sector, the training method was different from each other. The government training centres complied to the logbook requirements to provide PRP training. Near to two third of the participants under private hospital and community pharmacy reported there was no specific PRP training given to them, they learnt from the job and attended workshops occasionally. A minority of these training premises provided systematic training module to the PRPs, such as monthly presentation and case studies, departmental training and monthly medication review. For non-manufacturing sector, the PRP training was based on the projects and department's need such as presentation and workshop. Training schedule and checklist were prepared in certain departments but not all. In manufacturing sector, the standard operating procedure (SOP) training was usually given to the PRPs before on-the-job training. They needed to do presentation and handle different projects. PRPs from research and development started with the briefing for laboratory instruments and they were encouraged to participate in research related workshops and conferences.

Depend if you are in department such as pharmacovigilance and regulatory affairs where everything is capture and they want thing to be done in particular way then yes a lot of training. If is something more hey this is a question that we have do some research then no training will be provided.

(Non-manufacturing pharmaceutical industry, Interview 4, Line 168)

Incorporation of PRP training into university curriculum

Participants who agreed and disagreed to incorporate PRP training into undergraduate program were comparable. The benefit of incorporation was to provide pharmacy students with the pre-exposure of working environment and gain more practical experiences. Those with partial agreement or disagreement was mainly concerned with the students' capability, responsibility, knowledge and working attitude.

Incorporated is quite hard I would say because during university is still your learning time and I feel you should learn the most from the lecturer but during PRP time you are considered a working adult it comes with responsibilities with whatever you do so is not just about getting knowledge it comes with correct attitude.

(Government hospital, Interview 3, Line 133)

Preceptor's qualification

All the participants agreed the qualification of their preceptors was adequate to train a PRP. The preceptors were knowledgeable and are experienced in the relevant pharmacy field. However, there was one PRP from private hospital uncertain about the preceptor's qualification due

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to the lack of interaction between PRP and the preceptor.

For my preceptor I don't really deal much with her and so I'm not sure about her qualification but for my direct superior he is a very good teacher I would say, he actually taught me quite a lot of things.
(Private hospital, Interview 3, Line 214)

The present study examined qualitatively the experience, satisfaction and perception of Malaysian pharmacists towards their training with a total of 4 themes and 24 codes. The first theme was placement. According to the findings, the waiting time for the PRP placement in government sector has been largely reduced from average 18 months to 6 months or less after the service contract commenced. Participants prefer to receive notification from government regarding the intake schedule, so they will know how long they should be waited for the placement. The waiting period in private sector was comparatively shorter, the most was 6 months and the fastest was less than a month. Participants who preferred to do their PRP training in hospital settings predilected towards government hospital.The differences of PRP training module between government and private hospital are as shown in Table 2.

Table 2 : The comparison PRP training module between government hospital and private hospital (39-40)

Government Hospital	Differences	Private Hospital
8 weeks	Duration of ward pharmacy	4 weeks
Compulsory	Clinical Pharmacokinetics Services, Therapeutic Drug Monitoring Services (TDM) and Parental Nutrition Services (TPN)	Optional

In general, the clinical exposure and learning opportunity in private hospital are lesser compared to government hospital. Besides that, the pharmacists in private hospital are more focused on operational and pharmacy management. Participants also mentioned most of the doctors in private hospital are consultants which do not rely much on the pharmacist(21).They believed that clinical knowledge is the most valuable asset for pharmacists, much of gained are very useful even if the pharmacists decide to venture into different fields. Participants preferred a similar training duration for the clinical module as the government hospital in order to get more exposures and learning experiences. Further investigation is warranted to determine whether these optional training modules should be made compulsory. The health clinic PRP training module was not a necessity as reported by the participants because the knowledge and skill gained from hospital will be sufficient for them to manage the work tasks in health clinic.The training in health clinic was not sufficient for PRPs to handle work tasks in hospital.

The second theme was payment. In general, all the government training premises complied with the minimum allowance policy which provided at least RM2,600 per month to PRPs(22). The saturation in government sector consequently drove a large influx of qualified graduates into the private sector who competed for a limited number of training opportunities available. A more variable allowance was observed from one training premise to another and/or one training setting to another due to lack of standardized allowance scheme in private sector. According to our findings, all the participants under private sector, except research and development, received minimum allowance of RM 2,600 per month (22). However, not all the private training premises provided supplementary allowance to the PRPs, it is hugely depending on the company. There were participants who raised the concern that minimum allowance was not sufficient to cover their living costs and they suggested to provide supplementary allowance based on their needs. Those participants who practiced under research and development shared the same concern aforementioned. As this training module is relatively new compared to others, hitherto there is no any guideline on providing allowance to the PRPs. Further studies are required to determine if all the private training premises followed the minimum allowance as set in the government sector. Standardized allowance scheme should be implemented in all sectors with the consideration of the current living costs and to prevent the possibilities of PRPs being exploited in the process of securing any PRP placements.

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The third theme was working condition. The status of PRPs can affect their perception towards the PRP training. Inequality between PRPs and other employees were noticed in government hospitals. PRPs are required to do more basic work tasks and run errands that are irrelevant to their job scope, for example order food delivery for lunch talk. Besides that, PRPs are circumscribed from mingling with registered pharmacists due to the hierarchical structure as reported in government hospital. However, there was no issue of inequality in health clinic even if they are under the hierarchical structure. On the contrary, the status of PRPs in community pharmacy had a significant impact on their learning opportunities and job scope. If the PRPs are treated as employee, they will be working closely with the FRP sand able to involve more in management works. However, if the PRPs are treated as trainee, their job scope and learning opportunities are limited because they are not allowed to handle certain work tasks. The status of PRPs under research and development was different from all other settings, participants perceived themselves as a student during the 9 months research training as their job scope is totally different from other employees within the same organization. PRPs in pharmaceutical industry and private hospitals were treated as employee under training.

The last theme was training. The pharmacy curriculum in Malaysia is a combination of 3 aspects included pharmaceutical sciences, clinical pharmacy practice and research and development, the emphasis on one aspect or other varied from one pharmacy school to another. Based on the findings, the pharmacy curriculum provided sufficient basic knowledge for PRP training in different settings except for pharmaceutical industry. Majority of pharmacy schools emphasized more on clinical pharmacy practice than the pharmaceutical sciences. The modules covered under pharmaceutical sciences were mostly manufacturing related, in conjunction with non-availability pre-registration placements in non-manufacturing sector causing PRPs can only apply limited knowledge into their training. According to Kirby-Smith et al, this may affect the pharmacy graduate's perception towards pharmaceutical industry as they might have incomprehensive understanding of the wide range of career opportunities available and caused a substantial influence on loss of graduate pharmacists to the industrial sector (23). Furthermore, they have a common perception that their job scope will be clinical related as the undergraduate program is structured towards clinical settings. The pharmacy course content should be more balance between clinical pharmacy practice and pharmaceutical sciences in order to provide students with the necessary knowledge and skills for the pharmaceutical industry. There were participants suggested the provision of pharmacy placement in non-manufacturing sector would be beneficial for pharmacy graduates during the undergraduate program.

The inconsistency in the quality of PRP training was noticed across the practice settings. In addition, the discrepancies between preceptors in terms of knowledge, assessment, logbook requirement, and teaching style were mentioned by the participants from government hospitals. Similar findings was found in the previous study (15). It has recently been reported by local media that PRPs complained of lack of transparency in PRP evaluation and unavailability of feedback loop (24). PRPs were not given permission to review their marks and they couldn't learn from their mistakes and improve on weakness (24). The provision of PRP training is highly dependent on the preceptors and training centre. Although the criteria for pharmacists to become a preceptor was specified by PBM (25), there is no requirement to demonstrate their expertise in workplace assessment. Malaysia should implement a quality management system in PRP training likewise the Pre-Registration Pharmacist Scheme (PRPS) launched in 2006 by Scottish Government with the purpose of ensuring every PRP receive a high quality training opportunity and experience in all practice settings (26). According to Mills et al, a quality management system should encompass survey of PRPs and preceptors and visits to training sites (27-28). In Malaysia, the appraisal of preceptors by PRPs is optional and they are required to send the form separately from PRP logbook to PBM (29). The direct feedback of PRPs on their training is essential and should be made compulsory as to disclose problems and areas of PRP training needed for improvement (27-28). The mechanism for PRPs feedback can be implemented either through a national survey or locally implemented, or both (28). Another important element is regular site visits by the responsible bodies in order to ensure the quality of PRP training provided by the respective training facilities (27-28). At present, there are no studies to evaluate the current training workshop for preceptors, further studies are warranted in order to determine the needs of developing a structured training program (16) and performance management system for preceptor (27) as proposed by the previous studies. Otherwise the training would

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continue to be offered variously resulting in serious disparity (30) and negative learning experience (15, 16, 27, 30). The possibility to incorporate part of the PRP training into pharmacy curriculum was agreed and disagreed by the same number of participants. Some universities in United Kingdom offer a 5 year integrated pharmacy degree program which incorporates the pre-registration training into a single program of curriculum and training (31). The incorporation of PRP training into pharmacy curriculum guaranteed the pre-registration placements for all pharmacy students(32-34). Additionally, the employment rate and average starting salary for 5 years pharmacy graduates was higher than those 4 years pharmacy graduates (35). The incorporation of PRP training into Malaysia pharmacy education and extending the duration of undergraduate program to 5 years is possible, but from the student's point of view it could be challenging for them because they will need to pay tuition fees for one more year, instead of receiving allowance (32-36).

Even though the PRP training was stressful and challenging, the overall experiences of pharmacists toward their training were satisfactory and beneficial. However, there were areas of concern addressed by the participants from different settings. The 3 months hospital training should be reconsidered whether it should be put under research and development. The hospital training is more relevant to PRPs who study clinical postgraduate programs or those doing clinical research, by contrary, it is less relevant to those who study nonclinical courses. A number of participants urged for revision of PRP training module, including the logbook requirements, duration of training module and learning outcomes.The purpose of logbook is to serve as a guidance for PRPs to record their training and experiences (37), however the requirements of logbook is too overwhelming and repetitive, they tend to focus more on fulfilling the requirements than the objectives of PRP training. One of the interesting findings in this study was a participant who initially underwent the PRP training in community pharmacy and decided to change to hospital setting after 11 months of PRP training due to lack of knowledge and skills on prescription review. This participant suggested that PBM should focus less on hospital and develop a more precise community pharmacy PRP training module. This is important as the role of community pharmacists in Malaysia is evolving towards dispensing separation (38).

Pharmacists are required to fulfil specific requirements in order to secure a permanent post in government sector, however they may not immediately get the posts until there are vacancies (14). The first batch of contract pharmacists had completed the 2 years contract service in December 2018. Out of 500 pharmacists, 180 of them have been selected to receive the permanent post, however there are no any updates from the government regarding their postings (24). The local media also reported that PRPs urged for the transparency on the selection criteria of PRPs to receive the permanent post in government hospitals (24). Although PRPs are given briefing during the Mindset Transformation Programme (Program Transformasi Minda), but they need more detailed briefing on the second and third year of the contract post.

4. Conclusion

The participated pharmacists believed that PRP training was a necessary exposure to gain required experiences, despite all difficulties and challenges. The focus of government in the past few years was to resolve the saturation of PRP placement in government hospitals through the liberalisation of PRP training. However, more focus should be put on improving the inconsistency of quality in PRP training program in different practice settings in order to improve the experiences and perceptions of future PRPs toward their training.

Limitations

This study was mainly conducted in Klang Valley area and the findings may not be generalized in other regions of Malaysia. The majority of participants under government hospital setting were from larger hospitals, their experiences and perception might be different from those undergoing PRP training in smaller government hospitals. The suggestion of participants for the PRP training can be subjective, since this is a novel study the findings can be utilized as an indicator for further assessment on PRP training in different settings.

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Evaluation of Antibacterial Activity Against Multidrug Resistance (MDR) Bacteria by the Bark Fractions of *Canarium patentinervium* Miq.

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Abstract

Rapid emergence of antimicrobial resistance has become a concern worldwide. This is due to indiscriminate increase in bacterial adaptation towards conventional antibiotics. This has led to exploration of bioactive compounds from plants. *Canarium patentinervium Miq* belongs to the family of Burseraceae Kunth and genus *Canarium L*. This plant has been used traditionally in wound healing by indigenous people in Malaysia. This study aimed to search for an alternative antibiotic from medicinal plant and to provide ethnopharmacological evidence for its traditional use. The study aims to fractionate the ethanol extract of the barks of *Canarium patentinervium Miq* by using three solvents (petroleum ether, chloroform and water) and investigate its antibacterial activity against multidrug resistance (MDR) bacteria. Qualitative phytochemical analysis of the fractions of *Canarium patentinervium Miq* was examined for the presence of chemical constituents. The antibacterial activity of the fractions against reference bacteria, Methicillin sensitive Staphylococcus aureus (MSSA) ATCC 29213, Klebsiella Pneumoniae (K.Pneumoniae) ATCC 13883, Escherichia coli (E.coli) ATCC 35218 and clinical isolates, Methicillin resistant Staphylococcus aureus (MRSA), K.Pneumoniae, Acinetobacter Baumannii (A.Baumannii) were screened using disc diffusion method, minimum inhibitory concentration (MIC) assay and minimum bactericidal concentration (MBC) assays. Petroleum ether fraction exhibited bactericidal activity against MDR bacteria MRSA, MIC=0.125 mg/ml, MBC= 0.5 mg/ml (MBC/ MIC ratio= 4) and A. Baumannii, MIC= 1.0 mg/ml, MBC= 2.0 mg/ml (MBC/MIC ratio= 2). Water fraction displayed potent antibacterial activity against MDR strain of MRSA (MIC= 0.125 mg/ml) and A.Baumannii (MIC= 2.0 mg/ml) as compared to positive control respectively (vancomycin, MIC= 0.78 µg/ml and gentamycin, MIC= >25 µg/ml). The antibacterial activity of ethanol extract fractions of *Canarium patentinervium Miq*. bark supports the evidence of its traditional use and can be explored for bioactive compounds as antibiotic alternatives.

Key words: antibacterial, *Canarium patentinervium Miq.,* MDR

1. Introduction

Canarium patentinervium Miq belongs to the family of Burseraceae Kunth (torchwood family)and genus Canarium L. The genus *Canarium L* (derived from Malay name "kanari") comprises approximately 18 genera and 700 species.[1] The trees are mainly distributed in tropical Asia and the Pacific regions. The genus *Canarium* found in Asia Pacific region previously recorded for its usage in wound healing by the indigenous people of Malaysia. In previous studies, a number of chemical constituents such as tannins, flavonoids and sterols were presented in the ethanolic extract of leaves and barks of *Canarium patentinervium Miq*.[2] The extracts and pure chemical constituents derived from Canarium species have been revealed for antioxidant, antibacterial, anti-inflammatory, and antiacetylcholinerase properties.[2,3,4,5]

Study of plant secondary metabolites began the modern medicinal plants research in the early 19th century.[6] Bioactive compound has been reported to be therapeutically useful in treating disease.[7] Rapid emergence of multi drug resistance has become a concern issues in healthcare. Antibiotic has become less effective in treating infectious diseases due to increase of bacterial adaptation towards conventional antibiotics. Therefore, screening approach of a new effective medicinal leads from medicinal plants is essential to combat pathogens and to avoid the emergence of untreatable bacterial infections.[8,9]

Screening the properties of fractions of ethanol extract of the barks of *Canarium patentinervium Miq*., this study aims to investigate their phytochemical constituents and antibacterial activity to support the ethnopharmacological

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evidence in its traditional use and correlate between antibacterial activity to investigate their antibacterial activity against 3 reference strains (MSSA ATCC 29213, *K.Pneumoniae* ATCC 13883, E.coli ATCC 35218) and clinical isolates (MRSA, *K.Pneumoniae, A.Baumannii*).

2. Materials and Methods

Plant material collection and authentication

The barks of *Canarium patentinervium Miq* (CP).were previously collected from one individual tree from Bukit Putih, Selangor, Malaysia (3º5'24" N 101º46'0"E). The plant was identified with a herbarium sample (PID 251210-12) has been deposited in the Forest Research Institute of Malaysia (FRIM).

Fig 1. *Canarium patentinervium Miq.*

Fractionation of the ethanol extract of the barks of Canarium patentinervium Miq.

The extraction of this plant was previously done by supplier. 12.27 g ethanol extract of the bark of *Canarium patentinervium Miq* was dissolved in water. Then, it was sonicated by using asonicator for about 20 minutes to dissolve the extract completely. It was then subjected to fractionation by liquid-liquid partition using petroleum ether, chloroform and water solvents to yield the respective solvent fractions by using separating funnel.[4] It was concentrated with a rotary evaporator (Buchi, R-200 Switzerland). Water fraction was placed in the fridge at -80ºC for one day before undergoing freeze dryingusing a freeze dryer.

Phytochemicals screening

Qualitative phytochemical analysis was carried out to identify the presence of secondary metabolites in the plant fractions. Qualitative phytochemical analysis of the fractions to identify presence of secondary metabolites was determined as follows : [10,11,12]

Alkaloids test

Dragendorff's reagent : 200mg of the fraction was dissolved in 10ml of methanol and heated on a boiling water bath with 2 moles of hydrochloric acid, HCl (5ml). After cooling, the mixture was filtered and the filtrate was divided into two equal portions. One portion was treated with 1ml of Dragendorff's reagent. A prominent orange red precipitate indicates positive result.

Flavonoids test (Shinoda test)

4 mg fractions were dissolved in 0.2 ml ethanol and filtered. The filtrate was treated with a few drops of concentrated HCl and magnesium turnings (0.5g). The presence of flavonoids is indicative if pink or magenta-red color developed within 3 minutes.

Saponins test

The fraction was shaken vigorously to froth and then allowed to stand for 15-20minutes and classified for saponins content as follows: (no froth= negative; froth less than 1cm= weakly positive; froth 1-2cm high = positive; and froth greater than 2cm high = strongly positive).

Tannins test

About 1mg of fractions were dissolved in 1ml of hot distilled water and filtered. The solution was divided into two test tubes. To the first 0.9% sodium chloride (NaCl) solution was added, to the second 0.9% NaCl and 1% gelatin solution were added. Formation of a precipitate in the second treatment suggests the presence of tannins, which result in white precipitate supports this inference.

Sterols test (Salkowski Reaction)

4mg of fractions were dissolved in 0.2ml of chloroform and filtered. The filtrate was then added to 0.1ml of concentrated sulfuric acid, H_2SO_4. The presence of sterols is indicated by the 2 phase formation with a red color in the chloroform phase.

Cardiac glycosides test (Keller-Kiliani test)

A solution of glacial acetic acid (4.0ml) with 1 drop of 2.0% $FeCl_3$ mixture was mixed with 10ml aqueous plant extract and 1ml of concentrated H_2SO_4. A brown ring formed between the layers which showed the entity of cardiac steroidal glycosides.

Evaluation of antibacterial activity

Bacterial strains

For all the experiments, three different microbial (ATCC reference) cultures and three clinical strains were used. The clinical isolates of bacteria were obtained from University Malaya Medical Centre (UMMC). All bacteria strains were isolated from clinical specimens of hospitalized patients identified according to the Centers

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for Disease Control and Prevention / National Healthcare Safety Network (CDC/NHSN) criteria:[13]

Reference strains **:** MSSA ATCC 29213, *K.Pneumoniae* ATCC 13883, E.coli ATCC 35218

Clinical isolates **:** MRSA, *K.Pneumoniae* and *A.Baumannii*

Disc diffusion test

In vitro antimicrobial activity of fractions of ethanol extract of the barks of *Canarium patentinervium Miq* was studied against bacterial strains by using disc diffusion method, also known as Kirby-Bauer test following guidelines provided by the Clinical and Laboratory Standards Institute (CLSI).[14] In the present study, antibiotic was used as positive control (vancomycin used for gram positive bacteria, while gentamycin used for gram negative bacteria) and dimethylsulfoxide (DMSO) as negative control. Each standardized inoculum was adjusted at 0.5 Mcfarland standard / 625nm to yield 1 x 108cfu/ml by using Mueller Hinton broth (MHB). The inoculum was streaked onthe surface of agar plate. Paper discs were impregnated with the fractions already dissolved in pure DMSO and placed on the surface of inoculated agar with bacterial strain. Inhibition zones around each disc after an incubation of 37°C for 24 hours was measured and described as antibacterial activity.

Minimum inhibitory concentration (MIC) assay

MIC assay was described by Eloff[15] and performed in 96 wells plate by 2-folds serial dilutionfollowing the Clinical and Laboratory Standards Institute (CLSI) guidelines.[16] A serial dilution from the stock solution were ranging from 32mg/ml to 0.25 mg/ml using MHB. It aimed to evaluate antibacterial effects of the fractions.

Minimum bactericidal concentration (MBC) assay

MBC assay was performed using the method of Oztuk&Ercisli only for the susceptible bacteria from the MIC assay.[17] Only samples that have MIC values of lower or equivalent to 0.5mg/ml (strong inhibitors: PE and H2O fractions) were tested for the MBC values[18]. Ten microliters were taken from the well obtained from MIC value and two wells above the MIC well and spread on MHA plates. The number of colonies was counted after 18-24 hours of incubation at 37ºC.

Statistical analysis

All the results were expressed as mean ± SD. One way ANOVA and Tukey's test (Prism) were employed for the data analysis, when $p < 0.05$, the difference was considered significant.

3. Results and Discussion

Fractionation yield

Based on Table 1, H_2O solvent yielded the highest amount of fraction (77.0 %), followed by PE (18.1%) and $CHCl_3$ (1.5%).

Phytochemicals screening assay

Determination of the presence of secondary metabolite on the fractions was investigated by conducting phytochemicals analysis (alkaloids, flavonoids, tannins, saponins, sterols, and cardiac glycosides).

Investigation of secondary metabolites on phytochemicals analysis was described on Table 2. Presence of secondary metabolites varies between each fraction. Chloroform ($CHCl_3$) fraction only contains sterols. While petroleum ether (PE) fraction has all tested phytochemicals except for alkaloid and water (H_2O) fraction contains alkaloid, flavonoids, tannins, and cardiac glycosides. Previous study done by Mogana R *et al*. (2011) showed that ethanol extract of the barks of *Canarium patentinervium Miq* consists of flavonoids, tannins and sterols.

Antimicrobial susceptibility tests

Antibacterial activity of fractions of ethanol extract of bark of *Canarium patentinervium Miq* against reference bacteria strains (MSSA ATCC 29218, *K.Pneumoniae* ATCC 13883, *E.coli* ATCC 35218) and clinical isolates bacteria (MRSA, *K.Pneumoniae, A.Baumannii*) were evaluated by antibacterial susceptibility tests such as disc diffusion method, MIC and MBC assays.

For disc diffusion method, the results showed that H_2O and PE fractions have significant antibacterial activity with $p < 0.05$ against almost all the tested bacteria strains except for *K.pneumoniae* and *E.coli* (ATCC 35218) which shown in Table 3. H_2O and PE fractions displayed significant antibacterial activity against MDR bacteria, MRSA with zone of inhibition of 19.7 mm and 18.3 mm respectively and *A.Baumanii* with zone of inhibition of 10 mm and 9.7 mm respectively as compared to positive control. While, $CHCl_3$ fraction has least antibacterial activity among three fractions with sensitivity towards gram positive bacteria MSSA ATCC 29218 (inhibition zone= 6.83 mm) and MRSA (inhibition zone= 10.3 mm).

Only fractions (H_2O, PE) with higher activity were carried out MIC and MBC assay to determine the specificity of antibacterial activity. PE revealed bactericidal activity against MSSA ATCC 29213 (MBC/MIC=2), MRSA (MBC/MIC= 4), *K.Pneumoniae* ATCC

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13883 (MBC/MIC=1) and *A.Baumannii* (MBC/MIC= 2). PE displayed high sensitivity towards MDR bacteria such as MRSA (MIC= 0.125 mg/ml, MBC= 0.5 mg/ml, MBC/MIC= 4) and *A.Baumannii* (MIC= 1.0 mg/ml, MBC= 2.0 mg/ml, MBC/MIC= 2).

A.Baumannii was considerable resistant to gentamycin (MIC= >25µg/ml), however it showed sensitivity towards H2O (MIC=2.0 mg/ml) and PE fractions (MIC=1.0 mg/ml, MBC= 2.0 mg/ml, MBC/MIC = 2). This may be implied as a clear finding for a novel therapeutic choice to treat MDR *A.Baumanii infection.*

Table 1. Percentage of fraction yield of fractions of ethanolic extract of the bark of CP.

Solvents	Fraction
Petroleum ether (PE)	18.1
Chloroform ($CHCl_3$)	1.5
Water (H_2O)	77.0

Table 2. Preliminary phytochemical screening of the fractions of ethanolic extract of the barks of CP.

Tests	$CHCl_3$ Fraction	PE Fraction	H_2O Fraction
Alkaloids	-	+	-
Flavonoids	-	++	+
Saponins	-	++	+
Tannins	-	-	+
Sterols	+	++	-
Cardiac Glycosides	-	++	+

Keys: (+) present, (-) absent

Table 3. Antimicrobial susceptibility tests of the fractions of ethanolic extract of the barks of CP Miq.

Bacteria strains	Zone of inhibition (mm)				
	Plant fractions MBC,MIC values (mg/ml)			Control antimicrobial agents MBC,MIC values (µg/ml)	
	MBC/MIC ratio				
	$CHCl_3$	PE	H_2O	Vancomycin	Gentamycin
Gram positive MSSA (ATCC 29213)	6.83±0.29 NA	15.3±1.53[A] 1.0/0.5 2(+)	16.0±1.0[A] -/0.25 -	11.0±0.0 0.78/0.78 1(+)	NA
MRSA (MDR)	10.3±0.58[B] NA	18.3±1.15[C] 0.5/0.125 4(+)	19.7±1.15[C] -/0.125 -	10.3±1.15[B] 0.78/0.78 1(+)	NA
Gram negative *K.pneumoniae* (ATCC 13883)	- NA	13.7±1.53[D] 1.0/1.0 1(+)	14.0±1.0[D] -/1.0 -	NA	21.7±1.15 0.2/0.2[H] 1(+)
K.pneumoniae (MDR)	- NA	-	-	NA	17.0±1.0 0.39/0.2 2(+)
E.coli (ATCC 35218)	- NA	-	-	NA	15.0±1.0 6.25/1.56 4(+)
A.baumannii (MDR)	- NA	9.7±0.58[E] 2.0/1.0 2(+)	10.0±0.0[E] -/2.0 -	NA	Resistant -/>25[I] -

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Notes **:** $CHCl_3$: Chloroform fraction at the concentration of 0.6 mg/disc in DD assay, H_2O: water and PE: petroleum ether fractions at the concentration of 1 mg/disc in DD assay, vancomycin and gentamycin at 1 µg/disc in DD assay, vancomycin used for gram positive bacteria, gentamycin used for gram negative bacteria, -: no activity noted, that is, inhibition zone of 6 mm, NA: not applicable and MBC/MIC ratio ?4 = bactericidal (+) , >4 = bacteriostatic (-).

Data were obtained from triplicates experiments for disc diffusion method (n=9) and duplicate experiment for MIC assay (n=6) and represented as mean ± SD. Values with the same capital letter are considered as non-significant different (p>0.05) followed by One way ANOVA and Tukey multiple comparison test.

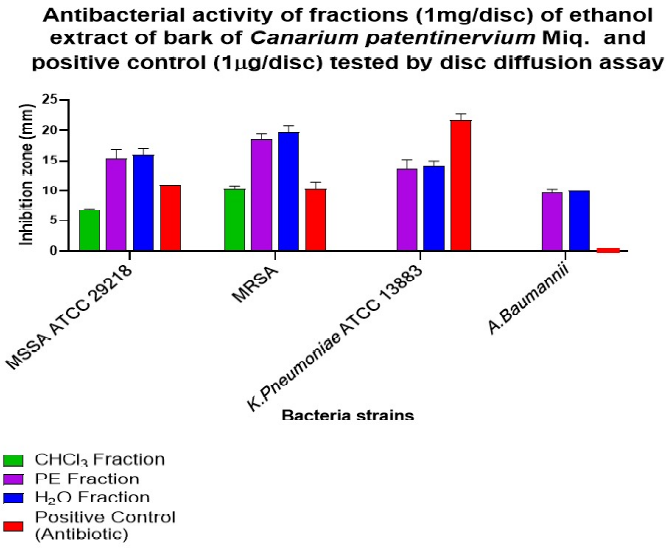

Fig 2. Antibacterial activity of fractions (1 mg/disc) of ethanol extract of bark of CP and positive control (1 µg/ml) tested by disc diffusion method

In the present study, it was noted that the highest percentage of fraction yield was with H_2O, followed by PE and the lowest with $CHCl_3$. The percentage of fraction yield may be associated with the polarity of solvents and characteristic of chemical constituents in the fractions.

The antibacterial activities of fractions of ethanolic extract of the CP barkswere evaluated by using disc diffusion method, MIC and MBC assays. The antibacterial activity of the fraction could be due to the presence of bioactive compounds that analyzed through phytochemical screening assay (Table 2). According to the preliminary study by Mogana R *et al*., the ethanol extract of leaves and barks and hexane extract of barks of CP revealed significant antimicrobial activity against gram positive bacteria *Staphylococcus aureus, Bacillus cereus*, MRSA and gram negative bacteria *Pseudomonas aeruginosa*.[2]

Phytochemical investigation of three fractions revealed the presence of bioactive compounds that serve as defense mechanism against microbes such as flavonoids (Xie YX *et al*., 2014)[19], saponins (Tagousop CN *et al*., 2018)[20], tannins (Akiyama H *et al*., 2001)[21], sterols (Kavita K *et al*.,2014 and Dogan A *et al*.,2017)[22,23] and alkaloids (Cushnie TPT *et al*., 2014)[24]. For flavonoid, evidences have shown that flavonoids exhibit inhibitory effects against the efflux pump of MRSA and against β-lactamases producing bacteria due to the structure of C6-C3-C6 skeleton possess antibacterial activity to defense wide range of pathogenic microorganisms.[19] According to Tagousop CN *et al*, saponins have synergistic effect in the combination of antibiotic which possibly associated with sugar moiety. This study revealed saponins possess highest inhibitory activity against S.aureus with less than 3 sugar moiety. Moreover, tannins owing antibacterial activity could be due to the existence of tannic acids which affect membranous structure of bacteria. According to Dogan A *et al*., the study reported that sterols have antibacterial activity can be explained based on peroxide and vinyl bonds in their structure.[23] Its mechanism may be correlated to the similarity of sterols in the bacterial cells. On the other hand, alkaloids such as indole alkaloids undergo dimerization to reveal antibacterial activity which possibly due to larger molecules of indole that are less prone to bacterial efflux. Hence, it can be concluded that fractions contain potent bioactive compounds with antibacterial activity.

Since the qualitative antibacterial activity of fractions of CP was determined by disc diffusion assay, therefore MIC and MBC assays were performed to evaluate the mode of antibacterial actions either bactericidal or bacteriostatic. According to Fabry *et al*., it was indicated that all plant extracts with MIC values < 8 mg/ml as active inhibitory agents. On the other hand, Van Vuuren was suggested medicinal plants with MIC $\leq$ 2 mg/ml were considered as active.[25] Hence, it can be considered that H_2O and PE fractions displayed high antibacterial activity against references bacteria and clinical isolated bacteria with MIC values ranged from 0.125 mg/ml to 2.0 mg/ml.

Generally, all fractions displayed antibacterial activity more pronounced to gram positive bacteria than gram negative bacteria. This is most probably due to the morphology of bacteria. Differences between gram positive and gram negative bacteria were associated with the composition of cell wall (Munyendo WLL *et al*., 2011).[26]

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Moreover, a clear indication was implied that *A.Baumannii* was susceptible to H2O (MIC= 2.0 mg/ml) and PE (MIC= 1.0 mg/ml) fractions. In the present study, there was considerable resistance to gentamycin with MIC value of >25 µg/ml. Fraction contains large amount of chemical constituents which may role in inhibiting the growth of *A.Baumannii* and as a valuable source with potent antimicrobial activity to reverse antibiotic resistance (Khameneh B *et al*.,2019).[27]

4. Conclusion

Fractions of ethanolic extract of the barks of *Canarium patentinervium Miq.* showed potent antibacterial activity possibly due to the presence of various bioactive compounds (tannins, flavonoids, saponins, sterols and alkaloids). This study revealed to support the evidence of its traditional use and explored for bioactive compounds as antibiotic alternatives. For future studies, it was recommended to carry out for bioassay guided isolation and identification of bioactive secondary metabolites as well as to study the mechanism of bioactive compounds action on MDR bacteria.

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Conflict of interest

The authors declare that they have noconflict of interests.

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Enzymatic and Non-enzymatic Antioxidant Potential of Methanolic Fractions of *Artabotrys suaveolens*

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Abstract

Artabotrys suaveolens is a tropical plant traditionally used for treatment of inflammation. The stem and leaves extract of Artabotrys suaveolens plant were investigated for 5-lipooxygenase inhibition (LOX) in both enzymatic and non-enzymatic invitro assays. The non-enzymatic antioxidant potential was determined using 1,1'-diphenyl-2-picrylhydrazyl (DPPH) assay and beta-carotene bleaching assay while the enzymatic antioxidant potential was measured by superoxide dismutase (SOD) assay. Nordihydroguaiaretic acid (NDGA) was used as positive standard. Phytochemical constituents of different fractions were determined. The chloroform fraction of the stem confronted antioxidant activity using DPPH assay (EC50: 7.89±0.50 µg/ml), beta-carotene bleaching assay (EC50: 8.04±0.65 µg/ml) and SOD assay (IC50: 13.83±0.35 µg/ml) with significant inhibition (IC50 value of 16.00±0.50 µg/ml) compared to NGDA (IC50 value of 55.80±1.00 µg/ml). The phytochemical analysis suggested the presence of alkaloids, cardiac glycosides and flavonoids in the stem of this plant. The significance of results supports the role of chloroform fraction from the stem of *Artabotrys suaveolens* as a lead compound with therapeutic usefulnessin treatment of inflammatory diseases.

Key words : *Artabotrys suaveolens*, 5-LOX inhibition, SOD assay, DPPH assay, beta carotene bleaching assay

1. Introduction

"Oxidative stress" is a situation when there is transient or chronic imbalance between reactive oxygen species (ROS) production and the ability of biological system to detoxify them through antioxidants (1). The modulation of redox state is crucial for cell viability, activation, proliferation, and organ function.At low concentration, ROS play vital role in body regulation and signaling process but at high concentration, they contribute to serious cellular damages (2).

In contrast, antioxidants are compounds that capable of donating their electron to stabilize these reactive species and reduce their harmful effects to human body (3). They are classified into two types; a) enzymatic which are endogenous antioxidants that present naturally in the body such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX) and glutathione reductase (GR) (4), and b) non-enzymatic which are further split into metabolic and nutrient antioxidants. Metabolic antioxidants are endogenous antioxidants produced from metabolism such as transferrin, glutathione, coenzymeQ-10, L-arginine etc. while nutrient antioxidants are exogeneous antioxidants obtained from diet or supplements such as vitamin C and E, trace metals (selenium, manganese, zinc), carotenoids, flavonoids, omega-3 and omega-6 fatty acids etc (3).

Both enzymatic and non-enzymatic antioxidants work together to ensure cell protection against oxidative damages resulted from reactive oxygen species (ROS) and reactive nitrogen species (RNS) [5,6].

5-lipooxygenase (5-LOX) is an important enzyme from arachidonic acid (AA) cascade [7], catalyses the synthesis of leukotrienes (LTs) [8]that are involved in the pathogenesis of various inflammatory diseases such as allergic rhinitis, asthma, cardiovascular diseases and certain types of cancer [9].

Currently, the only 5-LOX inhibitor approved for clinical uses is zileuton which is prescribed for treatment of asthma symptoms however; long term use of zileuton is associated with liver toxicity [10]. Hence, there is a need to search for new 5-LOX inhibitors with fewer side effects. Natural 5-LOX inhibitors from medicinal plants become increasingly important.

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In continuation of our natural and medicinal research programme on tropical rainforest plants [11,12], this study aims to investigate the inhibition of 5-LOX, the enzymatic and non-enzymatic antioxidant capacity of Artabotrys suaveolens plant.

A. suaveolens (from Greek, artao = supports and botrys = bunch of grapes and suavis = sweet) [13], is a plant belongs to the genus Artabotrys and family of Annonaceae. It is known by its local names, akarchenana and akarlarak in Malaysia [14] which is widely distributed in India (Nicobar Island), Malaysia, Philippines and Java, Indonesia [13]. A. suaveolens is traditionally used orally as emmenagogue. In addition, it is used to relieve fatigue after childbirth [15], to treat cholera [15] and to treat inflammation associated with enlarged spleen [16]. The above study is the first reported study on this plant and is documented by our team.

2. Materials and Methods

Materials **:** 1,1'-diphenyl-2-picrylhydrazyl (DPPH) from Calbiochem, beta-carotene, trolox, quercetin, dimethyl sulfoxide (DMSO) were purchased from R&M, tween 20, linoleic acid, superoxide dismutase (SOD) kit were bought from Cayman Chemical Company (Item number: 706002; batch number: 0526463), while nordihydroguairetic acid (NDGA) was purchased from Sigma Aldrich, enzyme 5-lipoxygenase enzyme (human recombinant) was purchased from Cayman Chemical Company and potassium phosphate buffer was from Sigma Life Science.

Plant materials **:** The leaves and stems of *Artabotrys suaveolens* were collected from a forest in Perak, Malaysia (4°46'N, 100°56'E). Plant identification was done by Forest Research Institute of Malaysia (FRIM). An herbarium sample has been placed at FRIM. The leaves and barks were air-dried and grinded into small particles using industrial grinder.

Extraction and fractionation **:** maceration was used for extraction through which the plant was soaked in a closed conical flask at room temperature [17]. Samples from leaves (2.7 kg) and stems (1.7 kg) were immersed in methanol (one part of the plant sample soaked in 3 parts of methanol) for 2 hours at 60oC water bath. Then, leaves extract was concentrated in a rotary evaporator (Eyela NVC-2200). The methanolic extracts of the leaves was indicated as LM (leaf methanolic crude extract) and the methanolic extracts of the stem was indicated as SM (stem methanolic crude extract). LM was partitioned with petroleum ether, chloroform and water to produce respective solvent fractions of different polarity using liquid-liquid partitioning technique. The same were repeated with SM and the fractions were labelled as LPE (petroleum ether fraction of leaves), LCL (chloroform fraction of leaves), LW (water fraction of leaves), SPE (petroleum ether fraction of stems), SCL (chloroform fraction of stems), and SW (water fraction of stems).

Phytochemical analysis : was performed as follows [18-20]

Alkaloids **:** 6 drops of Dragendorff's reagent were added into 1 ml of filtrate containing sample fractions with HCl. The presence of alkaloid was indicated by production of orange /brown precipitate.

Flavonoids **:** Shinoda test was done to test the presence of flavonoids. Few drops of concentrated HCl was added intofiltrate of sample fraction and magnesium ribbon. The presence of flavonoids was indicated by appearance of pink-tomato red colour.

Saponins **:** Frothing test was used. The presence of saponins was indicated by frothing of mixture containing sample fraction and distilled water which classified as follows: Negative results = no froth; Positive results = froth height (<1 cm: weak; 1-2cm: medium; >2 cm: strong).

Tannins **:** 5% w/v $FeCl_3$was added to filtrate containing sample fraction. The presence of tannins was indicated by the production of blue-black precipitate.

Sterols **:** Salkowski reaction was used. 1 ml of concentrated H_2SO_4 was added into the solution. The presence of sterols was shown by two phase formation with a red colour appearance.

Cardiac glycosides **:** In 2 ml plant fraction, glacial acetic acid, one drop of 5% ferric chloride (FeCl3) and concentrated sulphuric acid (H2SO4) were added. Reddish brown colour appears at junction of the two liquid layers and upper layer appearedwith bluish green, confirming the presence of glycosides.

Antioxidant capacity tests **:** Extract and fractions of leaves and stems were dissolved into DMSO prior to DPPH and beta-carotene assays at a stock concentration of 400 µg/ml and 2000 µg/ml respectively. Sample buffer supplied by SOD assay kit (Cayman Chemical) was used to dissolve plant samples for SOD assay to yield stock concentration of 1.25 mg/ml. All samples were then plated in a 96-well microtiter plate in different concentrations with serial dilution starting from 100 µg/ml to 3.125 µg/ml. Trolox, quercetin and standard SOD enzyme were

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used as positive controls. All three assays were performed using BMG LABTECH FLUO star Omega microtiter plate reader, connected to a computer equipped with (MARS Data Analysis Software 5.10 R2). GraphPad Prism version 7.04 were used to generate the EC50 and IC50 value. Three independent tests were carried out in triplicates for each sample in DPPH and beta-carotene assays whereas duplicates for each sample were done in SOD assay.

2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay **:** The DPPH assay was carried out according to Juan-Badaturuge method [21]. Aliquots of methanolic plant extract and fractions were dissolved in dimethyl sulfoxide (DMSO) at a stock solution of 0.4 mg/ml. Samplesat different concentrations were plated out in triplicates using a 96-well microtiter plate, prepared as serial dilutions from 100 µg/ml to 3.125 µg/ml. 1.183 mg of DPPH was added into 30 ml of methanol to obtain 0.1 mM of DPPH solution. The plate was covered by aluminium foil after adding the prepared DPPH solution, gently shacked for 2 min and kept in the dark for 30 minutes. Spectrophotometric measurements were done at 550 nm to obtain the percentage of decolourisation (colour change from deep violet to light yellow) and EC50 values were determined. Standard antioxidants (ascorbic acid, trolox and quercetin) were selected as positive control. The radical scavenging percentage for each sample was calculated using the equation:

$$\text{DPPH radical scavenging activity (\%)} = \frac{\text{Abs control - Abs sample}}{\text{Abs control}} \times 100$$

Where Abs control represents absorbance of DPPH radical + methanol; Abs sample represents absorbance of DPPH radical + sample extract /standard [21].

β-Carotene bleaching assay **:** This assay was carried out based on the method described by Habtemariam and Jackson [22]. Samples were plated out at different concentration in a 96-well microtiter plate. β-carotene solution was prepared based on the previous study described by R. Mogana et al. [23]. Additionally, sample plate for assay was prepared based on the previous study elaborated by R. Mogana et al [23]. In brief, 180 µL of the emulsion was pipetted into 20 µL of samples at different concentrations in the 96-well microtiter plate. The absorbance was obtained at 470 nm immediately and after 3h incubation at 50ºC against a blank consisting of emulsion without β-carotene by using a spectrophotometer. Trolox and quercetin were used as the positive standard. The antioxidant activity of the tested extract and fractions were evaluated in terms of bleaching of β-carotene using the following formula: antioxidant activity AA (%) = [1- (A0 - At)/(A'0 - A't)] × 100, where A0 and A'0were absorbances measured at zero time of incubation for the test sample and control, respectively; At and A'twere the absorbances measured in the test sample and control, respectively, after incubation for 3 hours.

Superoxide dismutase (SOD) assay **:** Cayman superoxide dismutase (SOD) assay kit [24] was used in the present study. The kit worked by mimicking the action of xanthine oxidase in the body which generated ROS during conversionof hypoxanthine to xanthine and then to uric acid. If the sample to be tested contained SOD enzyme, superoxide can be neutralized to produce hydrogen peroxide and oxygen. Rate of oxygen reduction had a linear relationship to xanthine oxidase activity and can be inhibited by SOD enzyme [25].

Failure of inhibition resulted in two simultaneous reactions **:** (1) oxidation of two superoxide anions to two molecular oxygen and (2) reduction of tetrazolium salt (colourless) to formazan dye (yellow). The IC50 (50 % inhibition activity of SOD or SOD-like samples) can be obtained by a colorimetric method. To perform the assay, 20 µL sample stock solution (2.5 mg/mL) was diluted in 20 µL of sample buffer to yield concentration of 1.25 mg/mL. The samples were plated in a 96-well microtiter plate in various concentrations ranging from 100 µg/mL to 3.125 µg/mL in which half of the concentration was reduced during each serial dilution. Standard SOD enzyme was also plated in different concentrations. 210 µL of radical detector and 20 µL of enzyme were added and incubated for 20 minutes at 37 ºC. All plant samples including standard SOD enzyme were diluted with sample buffer provided by the kit except petroleum ether and chloroform fraction of stems diluted with ethanol as both fractions are insoluble in sample buffer.

Absorbance of formazan dye was measured at the wavelength 450 nm using the BMG LABTECH FLUOstar Omega microtiter plate reader, linked to a computer equipped with MARS Data Analysis Software 5.10 R2. Recorded absorbance was proportional to concentration of superoxide hence absorbance has an inverse relationship with SOD activity.SOD enzyme provided in the kit was used as standard control. Sample well with buffer served as sample blank control to correct colour absorbance of samples. The rate of SOD inhibition and IC_{50} were determined. The IC_{50} was obtained from

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graph of SOD activity (%) against concentration of each plant sample.

***5-Lipoxygenase(5-LOX) inhibition assay* :** R. Mogana method for 5-LOX assay was used [25]. This method adapted the procedure outlined by Baylac and Racine [24] and Kamatouet.al. [26] with certain modifications. Human recombinant 5-LOX enzyme (from Calbiochem) was used. 100 U of enzyme was reconstituted using 4°C ice-cold buffer (potassium phosphate). DMSO was used to dissolve 20 µL of sample. Samples were then plated out in triplicates at various concentrations in a 96-well microtiter plate. Wells were added with 160 µL of 0.1M potassium phosphate buffer (pH 6.3) at room temperature. Then 20 µL of enzyme solution were added to all wells and mixture was agitated. 10 µL of linoleic acid was added at room temperature and incubated for 10 mins. Absorbance at 234nm was recorded. At this wave length, linoleic acid transformation (from 1-4-diene into 1-3-diene) can be detected. 5-LOX catalyses oxidation of unsaturated fatty acids containing 1-4 diene. Rates of reaction of samples was compared to blank using the formula below, percentage inhibition of enzyme was determined :

$$\text{Percentage inhibation of enzyme} = \frac{E-S}{E} \times 100$$

E = enzyme's activity without test sample

S = enzyme's activity with test sample

Similar with previous study, positive control Nordihydroguaiaretic acid (NDGA) was used.

***Statistical analysis* :** Data from three independent experiments were performed in triplicates (n=9), except for SOD assay which performed in duplicates (n=6). All results were expressed as mean ± SD and nonlinear best fit was plotted. Concentration-response curves were calculated using the Prism software package 7.04 for Windows, GraphPad Software, San Diego, California, USA, http://www.graphpad.com/ (GraphPad, San Diego, USA). One-way ANOVA with Tukey's multiple comparison tests was performed. Statistical significance is considered as $p < 0.05$.

3. Results and Discussion

Phytochemical analysis test

Alkaloids, cardiac glycosides, flavonoids, saponins, sterols/steroids and tannins were detected in the methanolic fractions of both leaves and stems of A. suaveolens(Table 1).

Antioxidant capacity tests

Antioxidant capacity of extract and fractions of A. suaveolenswere performed using both enzymatic (SOD)

Table 1 : Phytochemical analysis of fractions of leaves and stems *A. suaveolens*

Phytochemicals test	LPE	LCL	LW	SPE	SCL	SW
Alkaloids (Dragendroff's test)	-	++	+	+	++	-
Cardiac glycosides (Keller-Killani test)	+	+	-	+	++	+
Flavonoids (Shinoda test)	-	++	++	-	+++	++
Saponins (Froth test)	-	++	++	-	+	+
Sterols/Steroids (Salkowski's test)	+	-	-	+++	+	-
Tannins (Ferric Chloride test)	-	-	++	-	-	++

Key: +: low colour intensity, ++: moderate colour intensity, +++: high colour intensity. LPE: petroleum ether fraction of leaves, LCL: chloroform fraction of leaves, LW: water extract of leaves, SPE: petroleum ether fraction of stems, SCL: chloroform fraction of stems, SW: water fraction of stems

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and non-enzymatic (DPPH, carotene) assays. Non-enzymatic method involves two principle; hydrogen atom transfer (HAT) method by measuring the potential antioxidant activity to convert unstable free radicals to stable form through the mechanism of hydrogen atom donation [26] and single electron transfer (SET) method by measuring the capacity of the antioxidant to transfer one electron to reduce compound including metals, carbonyls and radicals [27]. β-carotene bleaching assay includes HAT method while DPPH uses both methods predominantly via SET method.

Stable free radical diphenyl-picryl-hydrazyl (DPPH) [28]and its specific absorbance properties can be used to estimate antioxidant activity [29]. The DPPH molecule is characterized as stable free radical by virtue of spare electron delocalisation over the molecule as a whole, so that the molecule does not dimerize [29]. The delocalisation give rise to deep violet colour. DPPH assay is based on the ability of antioxidant to reduce stable DPPH radical to form yellow coloured α, α-diphenyl-β-picryl hydrazine thus decolourising the deep purple DPPH methanol solution. In the β-carotene bleaching assay, the linoleic acid, which is a lipid, undergo reaction in the presence of reactive oxygen species (ROS) and oxygen (O_2) to produce an unstable peroxyl radical (LOO*). The peroxyl radical then reacts with β-carotene to produce a stable β-carotene radical which causes the bleaching of yellow solution. Competition reaction occurs with the presence of another antioxidant (sample) to react with LOO* which leads to slower bleaching of the solution detected at 470 nm spectrophotometrically [30].

Results of DPPH assay and the β-carotene bleaching assay are shown in Table 2. Fractions of both leaves and stems showed antioxidant activity.

DPPH assay results indicates that the LM confronted stronger antioxidant activity (EC_{50} values of 26.62±0.26 g/ml) compared to SM (EC_{50}> 100 g/ml). The antioxidant capacity for the leaf's fractions was as follows; LPE > LW > LCL. However, for stem fractions, the antioxidant capacity was as: SCL > SPE > SW. The beta-carotene bleaching assay also showed strong antioxidant activity possessed by the plant (Table 2). LM and SM showed antioxidant activity in beta-carotene bleaching assay with EC_{50} values of 2.37±0.50 g/ml and 6.11±0.45 g/ml respectively compared to positive. controls trolox (EC_{50} = 3.59 ± 0.61 g/ml) and quercetin (EC50 = 4.85±0.50 g/ml). The lipid peroxidation capacity of the leaves fraction was as follows: LW> LPE > LCL while the stems demonstrated activity of SCL> SPE & SW (EC_{50}>100 g/ml). In both assays SCL demonstrated strong activity compared to all other fractions.

Enzymatic antioxidant potential is commonly determined by measuring the SOD-like activity of a

Table 2 : Antioxidant and anti-inflammatory activitiesof leaves and stems methanolic extracts of *A. suaveolens*

Sample	DPPH assay, EC_{50} (µg/ml)	β-carotene assay, EC_{50} (µg/ml)	SOD assay, IC_{50} (µg/ml)	5-LOX inhibition, IC_{50} (µg/ml)
LM	26.62 ± 0.26	2.37 ± 0.50 [b]	53.50 ± 0.30	13.00 ± 0.70
LPE	20.60 ± 0.85	22.50 ± 1.31[d]	55.12 ± 0.42	22.70 ± 0.50
LCL	> 100	23.37 ± 0.61[d]	29.27 ± 0.81	26.90 ± 0.60
LW	32.90 ± 0.20	16.64 ± 0.6	19.67 ± 0.71	30.80 ± 0.60
SM	> 100	6.11 ± 0.45[c]	49.80 ± 0.90	11.20 ± 0.60
SPE	10.30 ± 0.21	> 100	22.40 ± 0.36	28.70 ± 0.70
SCL	7.89 ± 0.50 [a]	8.04 ± 0.65	13.83 ± 0.35	16.00 ± 0.50
SW	11.80 ± 0.40	> 100	3.25 ± 0.08	> 100
Trolox	7.33 ± 0.28 [a]	3.59 ± 0.61[b]	NA	NA
Quercetin	5.56 ± 0.61	4.85 ± 0.50[c]	NA	NA
SOD	NA	NA	0.20± 0.02	NA
NDGA	NA	NA	NA	55.80± 1.00

Key: LM: methanolic extract of leaves, LPE: petroleum ether fraction of leaves, LCL: chloroform fraction of leaves, LW: water extract of leaves, SM: methanolic extract of stems, SPE: petroleum ether fraction of stems, SCL: chloroform fraction of stems, SW: water fraction of stems, SOD: standard superoxide dismutase enzyme, NDGA: nordihydroguaiaretic acid, NA: not applicable. Data were express as mean ± SD, each perform performed as triplicates (n=9) in 3 independent experiments. Values with similar alphabet in the same column are not significantly different ($p < 0.05$) based on Tukey multiple comparison test.

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sample compared to SOD enzyme. SOD enzyme is a first line defence antioxidant that reduces and prevents generation of free radicals by catalysing the dismutation of superoxide anion to hydrogen peroxide [1]. In this study, xanthine oxidase is used to generate superoxide radical from oxygen. If the sample to be tested has SOD-like activity, superoxide can be neutralized to produce hydrogen peroxide and oxygen. If SOD-like activity is absent in the sample, superoxide will be reduced by tetrazolium salt (colourless) and colour of formazan dye (yellow) would be observed.

Methanolic extracts exhibited SOD-like activity with SM (IC_{50} = 49.80 ± 0.90 µg/ml) having higher activity than LM (IC_{50} = 53.50 ± 0.30 µg/ml), versus the positive standard SOD enzyme (IC50 = 0.20 ± 0.02 µg/ml). The leaves fraction enzymatic antioxidant activity was as follows; LW> LCL> LPE while the stem fractions activity was as follows; SW> SCL> SPE. The water and chloroform fractions of stem had the highest SOD-like activity (SW = IC_{50} 3.25 ± 0.08 µg/ml and SCL = IC_{50} 13.83± 0.35 µg/ml). SCL consistently had relatively higher antioxidant activity via all three assays. This effect is attributed to the presence of flavonoids, alkaloids and cardiac glycosides in these fractions [31-35].

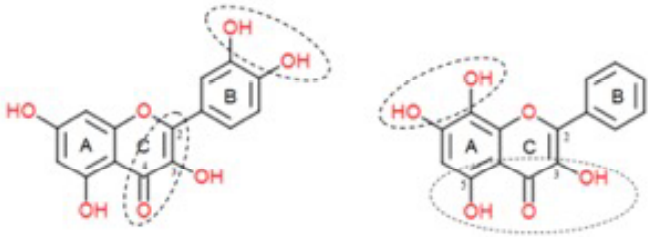

Fig. 1 : General structural requirement of flavonoid as antioxidant

Flavonoids have a general structure (Figure 1) consists of three 6-membered rings with one of them is a pyran ring having a carbonyl group [36]. Flavonoid works in various types of mechanism of action such as direct scavenging of ROS/RNS, chelating of trace metal ions involved in free radical production, inhibiting enzymes involved in production of free radicals, and regeneration of membrane-bound antioxidants. However, the consideration of primary antioxidant mechanism of flavonoid is hydrogen atom transfer [37]. Thus, o-dihydroxy substitution in B ring, C2-C3 double-bond, and C-4 carbonyl group in C ring are the structural activity requirements for hydrogen atom transfer (Figure 2).

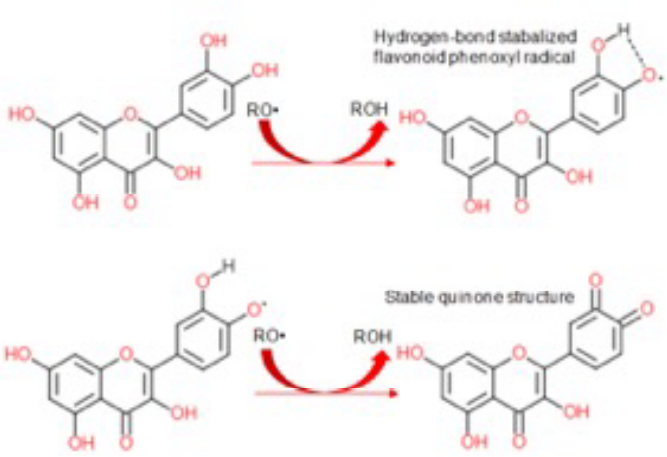

Fig. 2 : Mechanism of flavonoid as antioxidant via hydrogen atom transfer

In this study, SOD-like activity of SW and SCL was respectively superior than the others. High superoxide scavenging activity of water fraction could be contributed by polar compounds such as flavonoids [38-40] and tannins [41-43] where else the SOD-like activity of SCL could be due to the presence of cardiac glycosides, flavonoids and alkaloids [37] (Table 1). Various flavonoids which possess high SOD-like activity are highly polar (Figure 3) and it was suggested that high superoxide scavenging activity is contributed by hydroxyl group at C-3' in ring B and C-3 [44]. SOD-like activity of tannins is due to its polyphenolic structure with hydrophobic core which surrounded by polar compounds that form hydrophilic shell (Figure 4) [43], making tannin a water-soluble compound that remains in water fractions. However, because there is no recent structural activity relationship study of tannins on its superoxide scavenging activity, the specific structural features of tannin that contributes to its SOD-like activity is still not known.

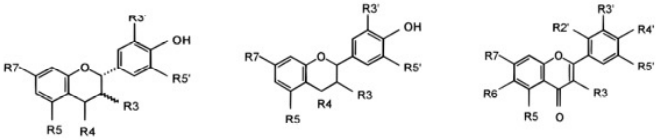

Fig. 3 : Chemical structures of flavonoids.

5-Lipoxygenase inhibition assay **:** The presence of 5-LOX inhibitor decreases the breakdown of linoleic acid into leukotrienes by 5-LOX enzyme. The change in the concentration of linoleic acid is detected using a spectrophotometer at 234 nm. Both LM (IC_{50} = 13.00±0.70 µg/ml) and SM (IC_{50} = 11.20±0.60 µg/ml) showed significantly higher 5-LOX inhibition as compared to positive standard NDGA (IC_{50} = 55.80±1.00 µg/ml).All fractions reported significantly higher inhibitory activity compared to NDGA except the water

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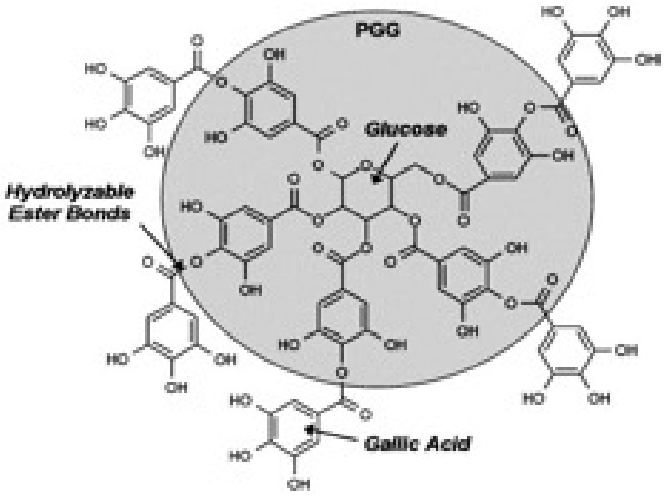

Fig. 4 : Chemical structure of tannin. Shaded area indicates the core structure of tannic acid - pentagalloylglucose. The hydrophobic core and hydrophilic shell are features responsible for antioxidant action of tannin.

fraction of stems as shown in table 2. The 5-LOX inhibition activity of the leaves were; LPE> LCL> LW while those of stems fractions were as follows; SCL> SPE> SW.This study focused on various antioxidant and anti-inflammatory assays include DPPH assay, beta-carotene bleaching assay, superoxide dismutase (SOD) assay and 5-LOX assay because of their close relationship in the anti-oxidative processes as the development of free radicals could be initiated by inflammation reaction [45] (Figure 5). linoleic acid (LA) is used in this study as it is structurally similar to arachidonic acid and is more stable [46]. A graphical abstract of this study is illustrated in Figure 6.

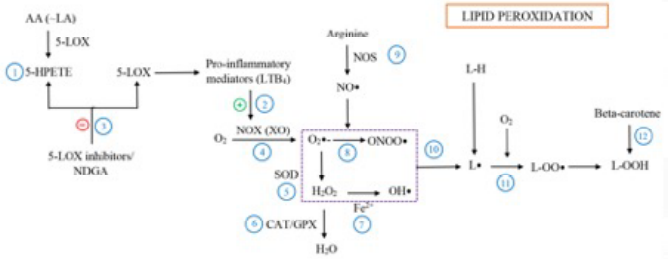

Fig. 5 : Relationship between assays of antioxidant and anti-inflammatory activities in this study

Key : AA: arachidonic acid, LA: linoleic acid, 5-LOX: enzyme 5-lipoxygenase, 5-HPETE: 5-(S)-hydroperoxyeicosatetraenoic acid, NDGA: nordihydroguaiaretic acid, LTB4: leukotriene B4, NOX: NADPH oxidase, O_2: oxygen, O_2·-: superoxide radical, XO: xanthine oxidase, SOD: superoxide dismutase, H_2O_2: hydrogen peroxide, CAT: catalase, GPX: glutathione peroxidase, H2O: water, OH·: hydroxyl radical, NOS: nitric oxide synthase, NO·: nitrite oxide radical, ONOO·: peroxynitrate, L-H: lipid membrane, L·: lipid membrane with free radical, L-OO·: lipid peroxyl radical, L-OOH: lipid membrane.

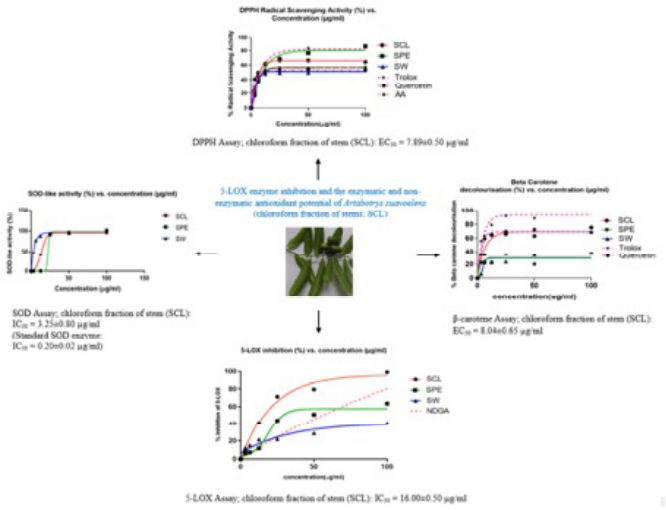

Fig. 6 : Graphical abstract of *A. suaveolens* 5-LOX inhibition activity

4. Conclusion

Artabotrys suaveolens is a tropical plant grows naturally in India, Myanmar, Thailand, Malaysia, Indonesia and Philippines. It istraditionally used for treatment of inflammation. The extractand fractions of *Artabotrys suaveolens* plant were investigated for 5-lipooxygenase inhibition (LOX) in both enzymatic and non-enzymatic invitro assays. The findings of this study support the antioxidant and anti-inflammatory potential of *A. suaveolens* as promising agents in treatment of oxidative stress-related diseases. Further isolation, characterization of the bioactive components and in vivo antioxidant research need to be carried out to further support the in vitro data.

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Conflict of interest

Authors have no conflict of interest.

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In silico Screening of Selected Flavanones for HMG CoA Reductase Inhibitory Activity

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Abstract

Hypercholesterolemia is one of the potential modifiable risk factors for cardiovascular diseases, the main leading causes of death globally. Statins (HMG CoA reductase inhibitors) are widely prescribed to keep serum levels of total cholesterol and LDL within the normal limit. Statins are generally well tolerated. However use of statins could lead to adverse effects such as elevated hepatic transaminases level, myalgia and increased risk of diabetes. These adverse effects could reduce patient compliance and results in poor therapeutic outcomes. Various flavanones are shown to possess anti-hypercholesterolemic effect in vitro, in silico and in vivo. In this present study, the binding energies of the selected flavanone compounds against HMG CoA reductase were determined through in-silico screening. The selected flavanones are eriocitrin, eriodictyol, hesperitin, hesperidin, neohesperidin, naringin, naringenin and narirutin. Atorvastatin was used as a positive control to validate the binding and to compare the binding energies of the selected flavanones. The structure of the human HMG CoA reductase (PDB ID: 1DQA) was downloaded from Protein Data Bank, whereas the structures of the flavanones were downloaded from ZINC database. All the compounds were prepared using AutoDock Tools 1.5.6. Then, they were docked against the human HMG CoA reductase using AutoDockVina 1.1.2 and Accelrys Discovery Studio 4.5. The interactions between flavanones and the protein were analyzed and their drug likeness was also determined. The binding energy of atorvastatin was found to be -8.0 kcal/mol. The flavanone glycosides, eriocitrin (-10.0 kcal/mol), hesperidin (-9.7 kcal/mol), neohesperidin (-9.5 kcal/mol), narirutin (-9.5 kcal/mol) and naringin (-9.1 kcal/mol) exhibited greater binding affinity towards HMG CoA reductase, as compared to atorvastatin. The flavanon glycone compounds, eriodictyol (-7.4 kcal/mol), hesperitin (-7.6 kcal/mol) and naringenin (-7.4 kcal/mol) exhibited lower binding energy than atorvastatin. However, they have total polar surface area (TPSA) lower than 140 $Å^2$ and do not violate the Lipinski's Rule of Five. Eriocitrin and hesperidin showed the estimated inhibition constant (Ki) in nanomolar range. Further in vitro and in vivo studies are required to analyze the correlation of these in silico findings.

Key words : Insilico docking, flavanones, atorvastatin, HMG Co-A reductase inhibitory activity, binding energy?

1. Introduction

Cardiovascular diseases are a group of disorders associated with the heart and blood vessels (1-4). According to the World Health Organization (WHO), the proportional mortality rate of cardiovascular diseases in Malaysia is 35% (5), which is the highest among the non-communicable diseases. As compared to the neighbouring countries, Malaysians also develop cardiovascular diseases at a younger age (58.5 years) than in Thailand (63.5 years) and in Singapore (68 years) (6). Apart from that, it is reported that the drug expenditures in Malaysia continue to rise in the recent years, with the highest increase in lipid modifying agents, antithrombotic agents and anti-diabetic agents for both public and private sectors (6). Atherosclerosis is the underlying pathology of cardiovascular diseases. It is often asymptomatic in the early phase and manifests as heart attacks and stroke in later years (2).

The most important risk factor for atherosclerosis is hypercholesterolemia (7), which is also one of the potential modifiable risk factors for cardiovascular diseases. The goal of current pharmacotherapy is to keep the serum levels of total cholesterol and LDL cholesterol within the normal limit. Statins are competitive inhibitors of HMG Co-A reductase enzymes which are widely prescribed in various countries for hypercholesterolemia (8). Generally, statins are well tolerated (9). However, recent studies reported that the use of statins could lead

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to an increased risk of diabetes, possibly due to decline in insulin synthesis (10). Other side effects of statins include elevated hepatic transaminases and myalgia (9, 11). The presence of these adverse effects could reduce patient compliance which lead to poor therapeutic outcomes. Hence, new drug development is necessary to minimize the adverse effects and to increase patient compliance. Flavonoids are naturally occurring low-molecular-weight polyphenolic compounds which are usually found in fruits and vegetables. Numerous studies had reported that flavonoids have broad spectrum of biological activities, including anti-inflammatory, anti-bacterial, anti-diabetic, anti-cholinesterase, antioxidant, hepatoprotective, anti-mutagenic, anti-carcinogenic and anti-hypercholesterolemia (12-15). Some studies showed that flavonoids such as epigallocatechin-3-gallate (EGCG) and curcumin exhibited HMG CoA reductase inhibitory activity in silico (16). Furthermore, some flavanones like hesperidin, hesperitin, naringin and naringenin are shown to improve the metabolism of cholesterol in vivo. The total cholesterol level was reduced upon administration of bergamot food extract which is rich in neoeriocitrin, naringin, neohesperidin, melitidin and brutieridin (13). Eriocitrin is also proved to potentially reduce the total cholesterol level by minimizing the accumulation of lipid in liver (17).

In this study, selected flavanones (eriocitrin, eriodictyol, hesperidin, hesperitin, neohesperidin, naringin, naringenin and narirutin) were tested against HMG Co-A inhibitory activity in silico and their structure-activity relationship was analysed.

2. Materials and Methods

The selected flavanones (Table 1) to be tested for the HMG CoA reductase inhibitory activity were eriocitrin, eriodictyol, hesperetin, hesperidin, naringenin, naringin, narirutin and neohesperidin. Atorvastatin was used as a positive control to compare the binding pose and energy of the selected flavanones. The structure of atorvastatin and the selected flavanones were downloaded from ZINC database(18).The structure of human HMG CoA reductase with a complex with atorvastatin (PDB ID: 1DQA)was downloaded from protein data bank (19). AutoDockTools 1.5.6 was used for preparation of protein molecule. Briefly, water molecules were removed, Kollman charges were added, polar hydrogens were added, non-polar hydrogens were merged and pdbqt file was generated. Similarly, the selected flavanones and atorvastatin were prepared by using AutoDock Tools 1.5.6 by adding polar hydrogens, accepting the proposed torsions followed by pdbqt file generation. The grid box was prepared with grid spacing at 1.0Å and grid points at center_x = -26.052; center_y = 7.737; center_z = 28.764; size_x = 40; size_y = 40 and size_z = 40 so that it covers the ligand and all the binding site residues in chain A of HMG Co-A reductase (Glu559, Cys561, Leu562, Ser565, Arg568, Arg590, Val683, Ser684, Asn686, Cys688, Asp690, Lys691, Lys692, Lys735, His752, Asn755, Asp767, Ser852, Leu853, Ala856 and Leu857) that are identified in a previous study (20). Chain A of HMG-CoA reductase (PDB ID: 1DQA) was selected for docking as all other chains are identical. The selected flavanone compounds were then docked against the human HMG CoA reductase by using AutoDockVina 1.1.2 by using all the default values (21). The results were analyzed by binding energies and root mean square deviation (RMSD) values. The estimated inhibition constant (Ki) was calculated for all the ligands by using a previously reported method by using the python script available at https://github.com/virtualscreenlab/Virtual-Screen-Lab/blob/master/DelG_to_Kd_converter.py (22). The resultant docking poses of the ligands and their interactions with the protein were analyzed using BIOVIA Discovery Studio Visualizer 4.5 (23). The drug likeliness of all the compounds was also analyzed and the best HMG Co-A reductase inhibitory compound was identified. The molecular properties of atorvastatin and the 9 ligands were calculated online by SwissADME (24).

3. Results and Discussion

Atorvastatin, as a positive control in this study, showed binding affinity of -8.0 kcal/mol towards HMG Co-A reductase (Table 2). Eriocitrin, hesperidin, neohesperidin, narirutin and naringin exhibited greater binding affinity compared to atorvastatin (Table 2). Among all the selected flavanones, eriocitrin showed the highest binding energy which was -10.0 kcal/mol, followed by hesperidin with -9.7 kcal/mol (Table 2). Neohesperidin and narirutin showed same binding energy, which was -9.5 kcal/mol, whereas the binding energy of naringin was -9.1 kcal/mol (Table 2). Hesperitin, naringenin and eriodictyol showed lower but comparable binding energies as compared to atorvastatin (Table 2). The binding energy of hesperitin was -7.6 kcal/mol, whereas naringenin and eriodictyol showed similar binding energy, which was -7.4 kcal/mol (Table 2). The inhibition constant (Ki) of all the ligands in μM range is given in Table 2. The binding interactions of atorvastatin and selected ligands are shown in 2D and 3D diagrams in Figures 1-6.Various molecular properties of all ligands are given in Table 2 and the drug likeness of all flavanone compounds were analyzed.

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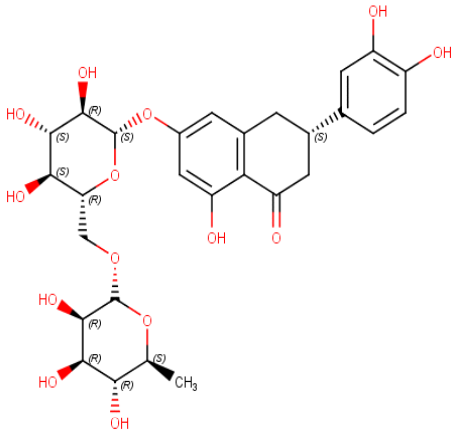
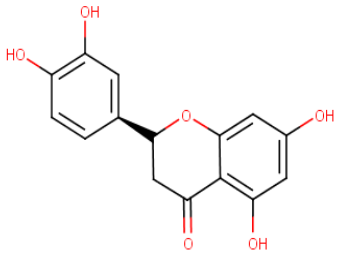
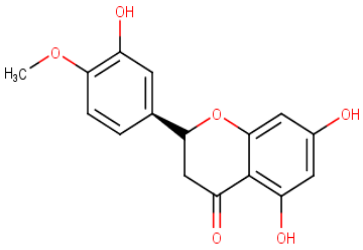
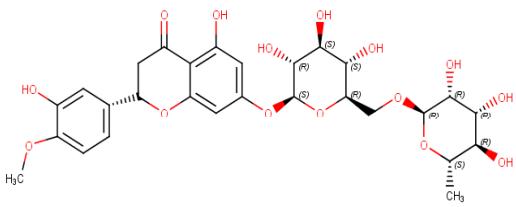
Table 1. List of ligands with their respective ZINC ID, name, IUPAC name and chemical structures

No.	ZINC ID	Name	IUPAC Name	Ligands
1.	8234294	S-Eriocitrin	(2*S*)-2-(3,4-dihydroxy-phenyl)-5-hydroxy-7-[(2*S*,3*R*,4*S*,5*S*,6*R*)-3,4,5-trihydroxy-6-[[(2*R*,3*R*,4*R*,5*R*,6*S*)-3,4,5-trihydroxy-6-methyloxan-2-yl]oxy-methyl]oxan-2-yl]oxy-2,3-dihydrochromen-4-one	
2.	58117	S-Eriodictyol	(2S)-2-(3,4-dihydroxy-phenyl)-5,7-dihydroxy-2,3-dihydrochromen-4-one	
3.	39092	S-Hesperitin	(2*S*)-5,7-dihydroxy-2-(3-hydroxy-4-methoxyphenyl)-2,3-dihydrochromen-4-one	
4.	8143568	S-Hesperidin	(2*S*)-5-hydroxy-2-(3-hydroxy-4-methoxyphenyl)-7-[(2*S*,3*R*,4*S*,5*S*,6*R*)-3,4,5-trihydroxy-6-[[(2*R*,3*R*,4*R*,5*R*,6*S*)-3,4,5-trihydroxy-6-methyloxan-2-yl]oxy-methyl]oxan-2-yl]oxy-2,3-dihydrochromen-4-one	

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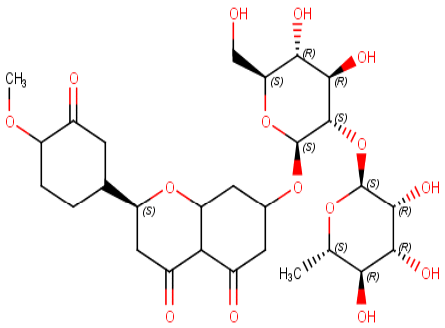
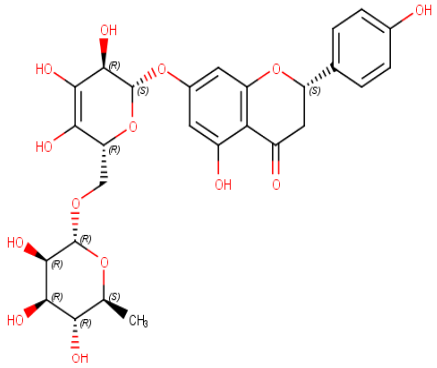
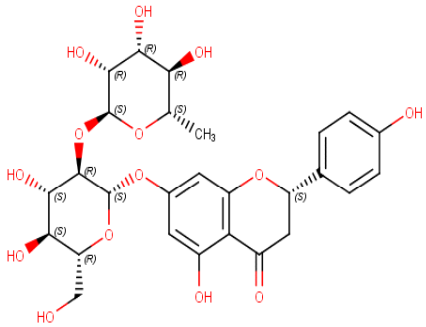
5.	8234302	S-Neohesperidin	(2*S*)-7-[(2*S*,3*R*,4*S*,5*S*,6*R*)-4,5-dihydroxy-6-(hydroxy-methyl)-3-[(2*S*,3*R*,4*R*,5*R*,6*S*)-3,4,5-trihydroxy-6-methyloxan-2-yl]oxyoxan-2-yl]oxy-5-hydroxy-2-(3-hydroxy-4-methoxyphenyl)-2,3-dihydrochromen-4-one	
6.	8234300	S-Narirutin	(2*S*)-5-hydroxy-2-(4-hydroxyphenyl)-7-[(2*S*,3*R*,4*S*,5*S*,6*R*)-3,4,5-trihydroxy-6-[[(2*R*,3*R*,4*R*,5*R*,6*S*)-3,4,5-trihydroxy-6-methyloxan-2-yl]oxy-methyl]oxan-2-yl]oxy-2,3-dihydrochromen-4-one	
7.	8143604	S-Naringin	(2*S*)-7-[(2*S*,3*R*,4*S*,5*S*,6*R*)-4,5-dihydroxy-6-(hydroxy-methyl)-3-[(2*S*,3*R*,4*R*,5*R*,6*S*)-3,4,5-trihydroxy-6-methyloxan-2-yl]oxyoxan-2-yl]oxy-5-hydroxy-2-(4-hydroxyphenyl)-2,3-dihydrochromen-4-one	

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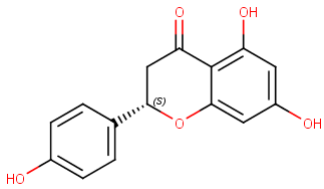
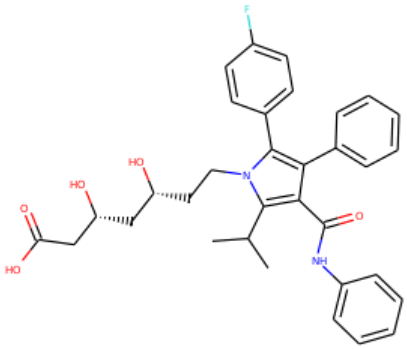
8.	156701	S-Naringenin	(2*S*)-5,7-dihydroxy-2-(4-hydroxyphenyl)-2,3-dihydrochromen-4-one	
9	3920719	Atorvastatin	(3*R*,5*R*)-7-[2-(4-Fluorophenyl)-5-isopropyl-3-phenyl-4-(phenyl-carbamoyl)-1H-pyrrol-1-yl]-3,5-dihydroxyheptanoic acid	

Table 2. Comparison of molecular properties and affinity of the ligands

S. No.	Ligands	Structure Analysis using Lipinski's rule of five				TPSA (Å²)	natoms	Binding Affinity (kcal/mol)	% ABS	Ki (Inhibition constant, µM)
		MW (Da)	HBD	HBA	log P					
1.	Atorvastatin	558.64	4	6	4.94	111.79	41	-8.0	70.4	1.302
2.	Eriocitrin	596.53	9	15	-1.28	245.29	42	-10.0	24.4	0.043
3.	Eriodictyol	288.25	4	6	1.45	107.22	21	-7.4	72.0	3.597
4.	Hesperitin	302.28	3	6	1.91	96.22	22	-7.6	75.8	2.563
5.	Hesperidin	610.56	8	15	-1.06	234.30	43	-9.7	28.2	0.073
6.	Neohesperidin	610.56	8	15	-1.02	234.30	43	-9.5	28.2	0.102
7.	Naringin	580.53	8	14	-0.87	225.06	41	-9.1	31.4	0.202
8.	Naringenin	272.25	3	5	1.84	86.99	20	-7.4	80.0	3.597
9.	Narirutin	580.53	8	14	-1.15	225.06	41	-9.5	31.4	0.102

Log P: octanol/water partition coefficient; TPSA: Molecular Polar Surface Area; natoms: Number of atoms; MW: Molecular weight; nrotb: Number of rotatable bonds; HBA: Hydrogen bond acceptor; HBD: Hydrogen bond donor; %ABS: Absorption

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Docking of selected flavanones towards HMG Co-A reductase Atorvastatin served as the reference standard compound in this study to validate and compare the binding energies of test compounds. According to the literature, the catalytic portions of HMG Co-A reductase are made up of amino acid residues 426-888(20, 25). Based on the 2D diagram in Figure 1, atorvastatin forms strong hydrogen bonds with Asn567, Arg568, Lys722, Ser865 and Cys561, with bond lengths of <3.0Å. It also forms weak hydrogen bonds with Ser565 and His866, with bond lengths of >3.0Å. Based on the previous studies, the weak van der Waals force interaction occurs between the atorvastatin and Leu562, His752, Ser852, Leu853, Ala856, Leu862, His869 and Tyr479 (26, 27). The amino acid residues around the binding pocket of atorvastatin found in this study were Asn567, Arg568, Lys722, Ser865, Cys561, Ser565, His866, Leu562, His752, Ser852, Leu853, Ala856, Leu862, His869 and Tyr479 (Fig. 1) and found to be similar to the previously reported studies (20, 26, 27). In general, the selected flavanones showed binding interactions with all these amino acid residues (Fig 3-6), except His752. Therefore, the selected flavanones may share a common binding method methodology with that of atorvastatin in HMG Co-A reductase.

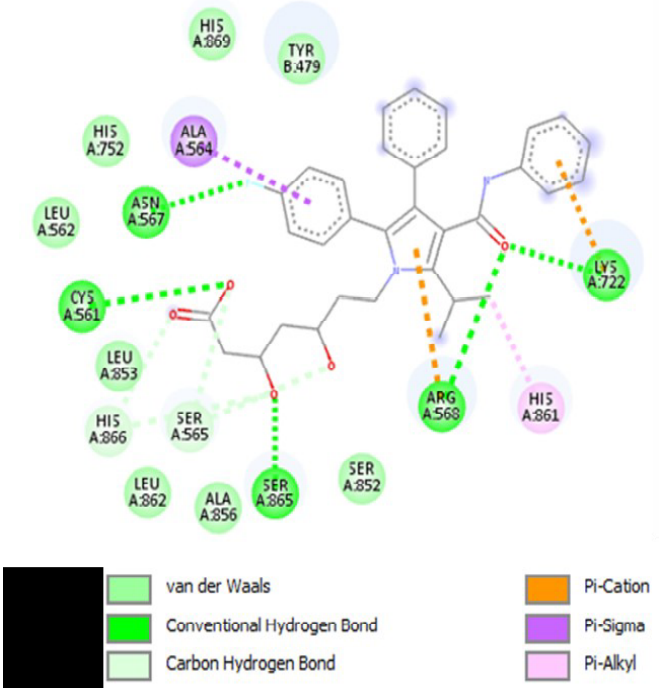

Figure 1. Interaction of HMG Co-A reductase with atorvastatin on 2D diagram

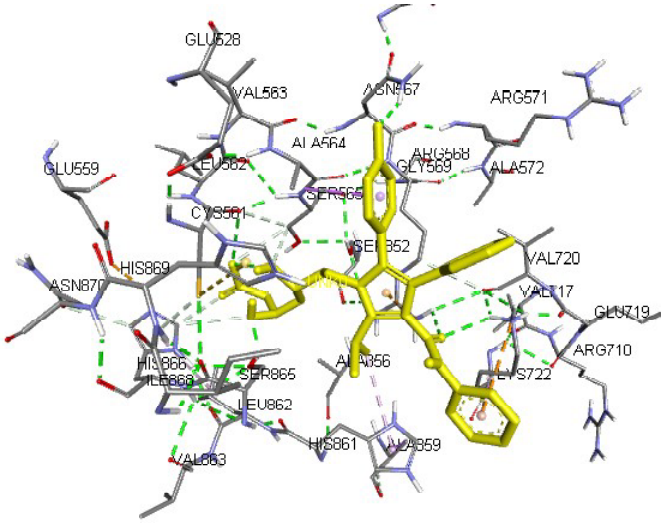

Figure 2. Interaction of HMG Co-A reductase with atorvastatin on 3D diagram

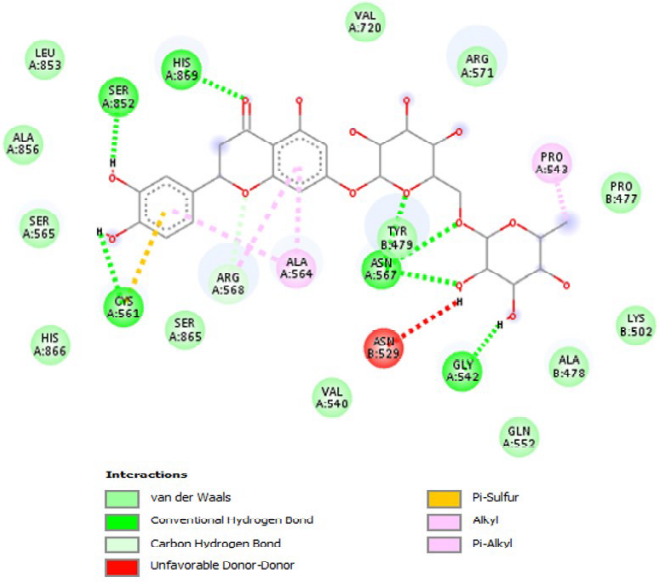

Figure 3. Interaction of HMG Co-A reductase with eriocitrin on 2D diagram

Atorvastatin, as a control in this study showed a binding affinity of -8.0 kcal/mol towards HMG Co-A reductase (Table 1). The flavanone-O-glycosides exhibited greater binding affinities than atorvastatin. They were eriocitrin (-10.0 kcal/mol), hesperidin (-9.7 kcal/mol), neohesperidin (-9.5 kcal/mol), narirutin (-9.5 kcal/mol) and naringin (-9.1 kcal/mol) (Table 1). In contrast, the aglycone flavanones exhibited lower but comparable binding affinities with atorvastatin. They are hesperitin (-7.6 kcal/mol), naringenin (-7.4 kcal/mol) and eriodictyol (-7.4 kcal/mol) (Table 1). The binding affinities of each flavanone differ from one another due to chemical structure differences.

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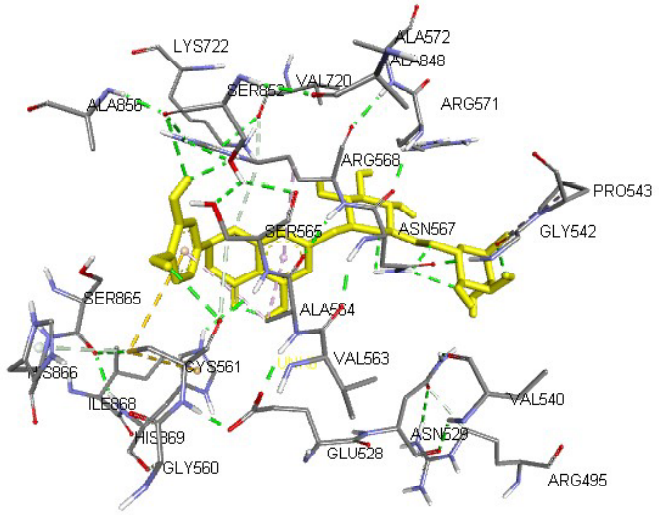

Figure 4. Interaction of HMG Co-A reductase with eriocitrin on 3D diagram

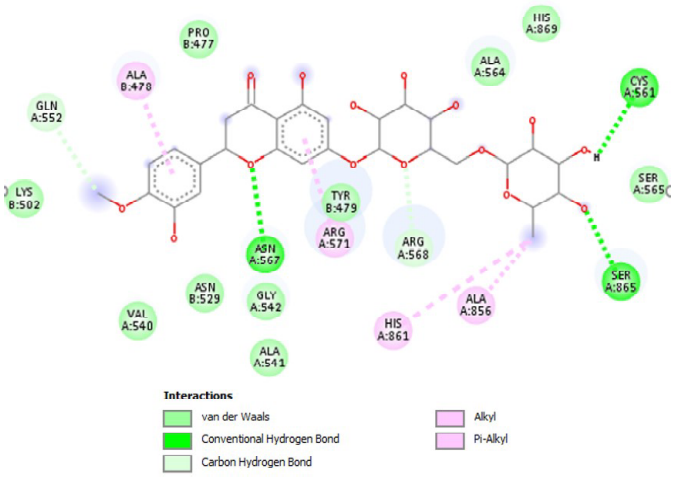

Figure 5. Interaction of HMG Co-A reductase with hesperidin on 2D diagram

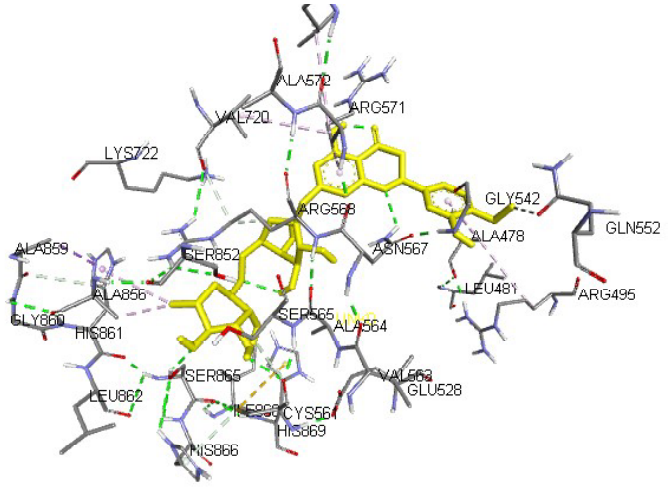

Figure 6. Interaction of HMG Co-A reductase with hesperidin on 3D diagram

The binding energies of eriocitrin, hesperidin, naringin and eriodictyol have been reported previously. According to Radhakrishnan et al., eriocitrin exhibits the highest binding energy towards HMG Co-A reductase, followed by hesperidin, naringin and eriodictyol, which are consistent with the results obtained in this study (28).

As in atorvastatin, all flavanones formed hydrogen bonds with Asn567 at the oxygen atoms in heterocyclic rings or in sugar moieties, except naringenin and eriodictyol (Fig 2-6). Naringenin formed weak van der Waals force with Asn567. Flavanones which interacted with Asn567, showed greater binding affinities. Hence, it is deduced that Asn567 plays an important role in the binding mechanism of flavanones towards HMG-CoA reductase. Eriocitrin (Fig. 3) and hesperidin (Fig. 5) which exhibited the highest binding affinities among all flavanones, also formed moderate to strong hydrogen bonds with Cys561 as in atorvastatin. It is proposed that the formation of these hydrogen bonds further stabilizes and enhances their bindings towards HMG Co-A reductase.

Arg568 plays an important role in the binding of statins towards HMG Co-A reductase (29, 30). All flavanone-O-glycosides formed weak hydrogen bonds with Arg568 (hydrogen bond lengths > 3.0 Å), similar to a previous study(31), except neohesperidin and naringin. The hydrogen bond interaction between O43 of neohesperidin and H21 of Arg568 is strong and mostly covalent, with a bond length of 2.37 Å(31). In naringin, the hydrogen bond is formed between Arg568 and the oxygen atom in heterocyclic ring. Naringenin is the only flavanone which formed hydrogen bond with Lys722, at a bond length of 2.66 Å. The hydrogen bond formation occurred between O1 of naringenin and NH of Lys722.

Eriocitrin, the disaccharide derivative of eriodictyol, consists of a dihydroxyphenyl ring substituted at position 2 of benzopyran-4-one ring. The hydroxyl groups in the dihydroxyphenyl ring of eriocitrin might have acted as hydrogen bond donors, which were responsible for hydrogen bond formation with Ser852 and Cys561 in the binding pocket of HMG Co-A reductase (Fig. 3). Besides, the hydrogen bond formation also occurred between His869 with the ketone group in the benzopyran-4-one

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ring, as well as between Asn567 and Gly542 with oxygen atoms in its sugar moiety (Fig. 3). Hence, the results suggest that the amino acid residues Asn567, Cys561, Ser852, His869 and Gly542 are important for hydrogen bond formation between eriocitrin and HMG Co-A reductase (Fig. 3 and Fig. 4).

Hesperidin differs from eriocitrin, as the hydroxyl group (-OH) at position 4' is replaced with a methoxy group ($-OCH_3$), which provides steric hindrance to the oxygen atoms and prevent hydrogen bond formation from taking place. Hesperidin formed hydrogen bonds with Asn567 and Cys561 as observed in eriocitrin, at benzopyran-4-one ring and its sugar moiety respectively (Fig. 5). Ser865 acted as hydrogen bond donor to oxygen atom in the sugar moiety of hesperidin, in forming a hydrogen bond. In contrast, Ser865 formed weak van der Waals force with eriocitrin (Fig. 3). There is no hydrogen bond formation with His869 and Gly542 in hesperidin, but weak van der Waals forces formed instead (Fig. 5). Hence, the results suggest that the amino acid residues Asn567, Cys561 and Ser865 are important for the hydrogen bond formation between hesperidin and HMG Co-A reductase.

Neohesperidin is a conformational isomer of hesperidin (32). The monosaccharides in neohesperidin are joined together through α-1,2 glycosidic linkage, whereas the monosaccharides in hesperidin are joined together through α-1,6 glycosidic linkage. Neohesperidin exhibited interactions which are quite similar to that of eriocitrin. Both the ligands formed hydrogen bonds with Asn567, Ser852, His869 and Gly542. As in atorvastatin, Arg568 acted as hydrogen bond donor in neohesperidin, forming strong and most likely a covalent hydrogen bond with its 3'-hydroxyl group. All other flavanone-O-glycosides formed weak hydrogen bonds with Arg568, except naringin. Hence, the results suggest that the amino acid residues Asn567, Ser852, Arg568, His869 and Gly542 are important for the formation of hydrogen bonds between neohesperidin and HMG Co-A reductase.

Naringin, which has a phenol group attached to the benzopyran-4-one ring, formed hydrogen bonds with Asn567, Arg568 and Tyr479. Both naringin and neohesperidin have disaccharides which are joined together through α-1,2 glycosidic linkage. The hydrogen bonds formed between Arg568 with naringin and neohesperidin occurred at the oxygen atoms in their phenyl rings attached to benzopyran-4-one. Hence, Arg568, Asn567 and Tyr479 are important for hydrogen bond formation between naringin and HMG Co-A reductase.

Narirutin is the conformational isomer of naringin(33). The monosaccharides in narirutin are joined together through α-1,6 glycosidic linkage. Narirutin formed hydrogen bonds with Asn567 at the benzopyran-4-one ring, and also with Lys474 and Gln552 at the oxygen atoms in its sugar moiety. The results indicate that Asn567, Lys474 and Gln552 are the amino acid residues in the binding pocket of narirutin in HMG Co-A reductase.

For aglycone flavanones, hesperitin, with methoxy group ($-OCH_3$) substituted at C4' of the phenol ring only interacted with Asn567 to form hydrogen bond. The presence of the $-OCH_3$ group is thought to provide steric hindrance to the oxygen atoms and prevent binding interactions. As in atorvastatin, hesperitin interacted with Tyr479, Ser852, Leu853 and Leu862 through weak van der Waals force. Eriodictyol formed hydrogen bond with Glu730, Glu782 and Asn734 at the hydroxyl groups in dihydroxybenzene rings. Glu730 and Glu782 served as hydrogen bond acceptors, while Asn734 served as hydrogen bond donor. Naringenin has a phenol ring attached to the benzopyran-4-one. As in atorvastatin, it formed a hydrogen bond with Lys722 at the ketone group in benzopyran-4-one ring, and interacted with Asn567 through weak Van der Waals force. It also formed hydrogen bond with Glu719 at the hydroxyl group in benzopyran-4-one ring.

Drug likeness analysis

Lipinski's rules of 5 (Lo5) is used to evaluate the drug likeliness and pharmacokinetics (ADME - absorption, distribution, metabolism and excretion) of drug substances, as well as to determine whether the drug is biologically active. The components of Lo5 include (a) molecule with molecular weight less than 500 Dalton, (b) no more than 5 hydrogen bond donors, (c) no more than 10 hydrogen bond acceptors and (d) octanol-water partition coefficient log P is not greater than 5(34-36).

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Any drug substance which violates more than one of the Lipinski's rules is said to possess poor solubility, absorption and permeability (34-36).

Based on the results obtained in Table 2, the molecular weight of atorvastatin is more than 500 Da. It also has 4 hydrogen bond donors, 6 hydrogen bond acceptors and a log P value of <5. Since it does not violate more than one Lo5, it is assumed to have good solubility and permeability. In contrast, all flavanone-O-glycosides (eriocitrin, hesperidin, neohesperidin, naringin and narirutin) which exhibited greater binding affinities than atorvastatin have violated more than one of the Lipinski's rules. Their log P values were in accordance with Lo5, but they have molecular weights of >500 Da, more than 5 hydrogen donors and more than 10 hydrogen acceptors. However, Lo5 is not applicable to substrates transported through active transporters (36). Hence, further in vitro studies should be carried out in the future to investigate the drug-likeliness of flavanone-O-glycosides and their transport mechanisms in vivo.

On the other hand, aglycone flavanones which exhibited lower but comparable binding affinities have molecular weights of <500Da, which are 288.25Da, 302.28Da, 272.25Da for eriocitrin, hesperitin and naringenin respectively. Furthermore, they have < 5 hydrogen bond donors, < 10 hydrogen bond acceptors and octanol-water partition coefficient log P <5. Hence, the aglycone flavanonesobey the Lo5, which indicates that they are drug-like substances, possess desirable pharmacokinetic properties and most likely biologically active.

The topological polar surface area (TPSA) refers to the surface of polar atoms, which correlates with the passive molecular transport across membranes. TPSA is often used to predict intestinal absorption and penetration through blood brain barrier(37, 38). A TPSA of <60Å^2 indicates that the compounds will be completely absorbed (39, 40), whereas a TPSA of <140Å^2 indicates that the compounds are more likely to have good permeability (37, 41, 42). Atorvastatin has TPSA <140Å^2, and thus expected to possess good absorption in the small intestine. The TPSA of all flavanone-O-glycosides are greater than 140Å^2 (eriocitrin = 245.29 Å^2, hesperidin and neohesperidin = 234.30 Å^2, naringin and narirutin = 225.06 Å^2), whereas the TPSA of all aglycone flavanones are less than 140 Å^2 (eriodictyol = 107.22 Å^2, hesperitin = 96.22 Å^2, naringenin = 86.99 Å^2). Therefore, eriodictyol, hesperitin and naringenin are expected to have good permeability across the intestinal membrane. However, these theoretical predictions need to be correlated with actual data as contrasting data has been reported on atorvastatin's intestinal absorption and bioavailability (43).

TPSA can also be used to calculate the percentage of intestinal absorption (%ABS) by %ABS = 109 - [0.345 x topological polar surface area (TPSA)], according to the method of Zhao et al (37, 44). Compounds, which have high TPSA values are expected to have low absorption (%ABS). Hence, based on the results obtained, all flavanone-O-glycosides are expected to have poor intestinal absorption, whereas all aglycone flavanonesare expected to have great intestinal absorption (eriodictyol = 72.0%, hesperitin = 75.8%, naringenin = 80.0%), owing to their good permeability across the intestinal membrane.

4. Conclusion

Based on the findings of this study, all flavanone glycosides (eriocitrin, hesperidin, neohesperidin, narirutin and naringin) exhibited higher binding affinities towards HMG Co-A reductase when compared to atorvastatin with eriocitrin having the highest binding affinity. Eriocitrin and hesperidin showed the estimated inhibition constant (Ki) in nanomolar range while other compounds showed in micromolar range. Drug likeness analysis indicated that all the flavanone aglycones have favorable absorption property when compared with flavanone glycosides. Further in vitro and in vivo studies are required to analyze the correlation of these in silico findings.

Conflict of interest

The authors declare that they have no conflict of interest.

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Gamification Technique to Estimate Mini Mental State Examination Scores : A Validation Study

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Abstract

Current cognitive screening methods are less interactive, costly, time consuming and require trained staff to perform the task. The goal of this study was to demonstrate the validity of a freely available game-based instrument for the self-assessment of cognitive function. We conducted a cross-sectional observational clinical study on 47 participants who were 18 years or older, diagnosed with neuromedical illnesses, and without physical and psychiatric illness. The result showed that a total, 25 females and 22 males between the ages of 18 and 78 years were included. Our assessment tools included the MMSE conducted and scored by physicians and the Holey Moley freely available game. Participants received instructions and brief practice prior to the assessment. The actual assessment was conducted after the hands-on practice, and MMSE and game scores were recorded. MMSE scores ranged from 9-30 with 12 participants classified as having impaired cognition. The Holey Moley game scores ranged from 7-113. Our experiment results showed a normalised root mean square error of 8.1% between the actual and estimated MMSE scores. There was a significant positive correlation between MMSE and game score ($r= 0.92$, $P <0.01$). The feely available Holey Moley game is a promising instrument for cognitive screening in clinical settings. This work demonstrates the feasibility of utilising games for cognitive screening in a health care environment.

Key words : Cognitive screening, Mobile games, Cognitive screening tools, Feasibility

1. Introduction

Cognition refers to high-order processes, primarily involving the cortical structures of the brain, that program adaptive behaviour, solve problems, memorise information, and focus attention (1). Antiepileptic drugs (AEDs) are known to affect cognitive function by suppressing neuronal excitability or enhancing inhibitory neurotransmission (2). Thus, the main cognitive effects of AEDs are impaired attention, vigilance, and psychomotor speed. Standard cognitive assessment tools such as the Montreal Cognitive Assessment (MoCA), and the Mini-Mental State Examination (MMSE) are used in healthcare settings (3,4). The Bahasa Malaysia version of the MoCA (MoCA-BM), a validated translated version of the MoCA, is also available for cognitive assessment among Malaysian communities (5). These screening tools are paper-and-pencil based and require administration by a trained healthcare professional. However, difficulties in completing the MoCA have been reported due to the inability of some patients to hold a pencil (6). A similar issue was also reported for the MMSE, particularly for the writing and drawing tasks (7). The Early Dementia Questionnaire (EDQ) is a promising alternative to the MMSE for screening of cognitive assessment in primary care (8).

Current cognitive screening methods are minimally interactive, providing patients with less motivation to complete the assessment (9). Software suites such as CogTest, the Cambridge Neuropsychological Test Automated Battery, Oxford's Cancellation Tools, Cognifit, and Lumosity offer computerised versions of traditional cognitive tests (10-14). These tools can also be utilised for brain training and to improve cognitive function. Additionally, WESIHAT 2.0 is a suitable tool for educating elderly people regarding lifestyle modification approaches to aid in slowing the progression of cognitive decline (15). Validation issues may arise when transitioning the test to a computer medium, and there is a potential for lack of motivation while executing moderately boring tasks on a computer. Accurate screening of cognitive function can aid in differentiating between age-related and abnormal cognitive decline (16). Moreover, conventional screening methods are costly, time consuming and require highly skilled staff (17).

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To make cognitive assessment more enjoyable and entertaining, gamification can be used to improve user experience and engagement (18). Games can be entertaining, helping the user to relax cognitively and mentally (19). Additionally, these games can offer valid cognitive assessment without loss of predictive validity in a cost-effective manner (20). Several games have been specifically developed for use in health care, such as to manage juvenile diabetes, asthma, and depression (21-24). Another study reported the use of a technique involving a game that focuses on improving cognitive function (25). Games allow more effective monitoring of cognitive status and allow changes in cognitive status to be detected more quickly (26). A Canadian study developed a game intended for cognitive assessment, "Whack-A-Mole", that showed significant correlation with the MMSE (27). These results cannot be generalised as the study included only elderly patients (70 years or older). Similarly, a study by Manera et al. using a cooking game was also performed on a very focused population, including only patients with dementia (28). Thus, validated game-like screening tools that can be completed rapidly and independently by a broad range of adults with varying cognitive abilities are warranted. The goal of this study was to validate a game-based cognitive assessment delivered on tablet technology to a clinical sample of patients with neuromedical illnesses.

2. Materials and Methods

Study population and sampling method

The patients were recruited from the neurology clinic at the tertiary care hospital in Malaysia. Data was collected using simple random sampling. List of patients was obtained from the clinical appointment record and the patients were randomly called in the physicians room. Patients were recruited based on the following criteria.

Inclusion criteria

Patients with epilepsy, Parkinson's Disease, stroke and Alzheimer's disease, who were 18 years or older and taking neurological related medications for at-least 6 months,

Exclusion criteria

Patients with physical or psychiatric illness such as schizophrenia and major depression, critically ill patients and those who refused to participate in this study and patients not fit to be interviewed determined by the physicians were excluded.

Sample size

A total of 47 participants were included, which was higher than the estimated sample size. The minimum estimated sample size, calculated based on a moderate correlation at $r = 0.5$ with α of 0.05 and study power of 80%, was 29 participants (29).

Assessment tools

Our assessment tools included the MMSE, Holey Moley freely available game, and a 10-inch mobile tablet.

Game selection

Holey Moley, a mole whacking game offered by Refresh Creations and developed by Ryan Carso (30) was selected because it is free to use on Android and iOS operating systems, and comparable to the Whack-a-Mole game used by Tong et al. for cognitive assessment (26).

The Whack-a-Mole and Holey Moley game differ in target and distractor characters. The comparison between the game variants are summarised in Table 1.

Table 1: Summary of comparison between game variants.

Parameters	Games	
	Whack-a-Mole	**Holey Moley**
Target	Mole and Squirrel	Mole and Mole with Hat
Distractor	Rabbit, Butterfly, Blue Mole	Bomb
Game duration	60 sec	60 sec

Based on a literature review (20,26-27) , a checklist was created to compare the Holey Moley game with different assessment methods such as the MMSE, the MoCa, the Whack-a-Mole game, Lumosity, and Cognifit. The parameters compared were focus, speed, memory, problem solving, coordination, and language. Time needed to complete the task and cost were also compared. A panel of 5 experts, consisting of lecturers and healthcare practitioners, reviewed the checklist and compared assessments. Their evaluations were similar. Based on their scoring, the Holey Moley game was chosen as it is free, time efficient, and covers the majority of parameters (focus, speed, memory, coordination) in cognitive assessment. The expert panel's scoring of the checklist is summarised in Table 2.

Ethical considerations

Ethical approval was obtained from the ethical committee of Universiti Kebangsaan Malaysia Medical Centre, reference no. UKM PPI/111/8/JEP-2017-138. Written informed consent was obtained from all patients before participation in the study. Patients who refused to participate in this study were excluded.

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Table 2 : Checklist for the comparison of cognitive assessment methods.

Items	Parameters							
Assessment Methods	Time Needed	Cost	Focus	Speed	Memory	Problem Solving	Coordination	Language
MMSE	10 min	$1.6/test	5/5	0/5	5/5	5/5	0/5	5/5
MoCA	10-15 min	Free	5/5	0/5	5/5	5/5	0/5	5/5
Whack-A-Mole game	60 sec	N/A	5/5	5/5	5/5	0/5	5/5	0/5
Holey Moley	60 sec	Free	5/5	5/5	5/5	0/5	5/5	0/5
Lumosity	>10 min	$15/ month	5/5	5/5	5/5	0/5	5/5	0/5
Cognifit	>10 min	$19.95/ month	5/5	5/5	5/5	5/5	5/5	0/5

*Value in table is number of people in agreement/total no of people in panel

Data collection

Data collection included age, sex, race, education, comorbidities, MMSE score, and game score. The MMSE was conducted and scored by physicians who were blinded to game score. After completion of the MMSE, researchers explained the graphical interface of the game and gave instructions to the participants on how to play the game. Participants were given time to interact with the tablet, involving hands-on practice for up to 5 minutes to verify if they had any difficulty in understanding the graphical and/or textual information provided on the screen. The actual assessment was conducted for 1 minute after the hands-on practice. Participant scores on the MMSE, and the Holey Moley game were recorded for correlation analyses and validation.

Statistical analyses

Statistical analyses were performed using the IBM SPSS Statistics Version 23. Frequencies and percentages were used for categorical variables, and descriptive statistics, including mean and standard deviation (SD), were used for continuous variables describing the study population. The MMSE cut-off score for cognitive impairment is 23 (31). The game cut-off score for cognitive impairment was determined by ROC curve, and sensitivity and specificity tests. The trade-off value from the ROC curve was used to categorise the game values into cognitive impairment, and normal cognition. The root mean-square error (RMSE) and normalised RMSE (NRMSE) were used to evaluate the estimation performance. High R2, eg R2 > 0.6 and RMSE less than 10% ensures the model fits the data well (32).

3. Results and Discussion

In total, 25 females and 22 males between the ages of 18 and 78 years were included in the study. The MMSE scores ranged from 9 to 30, with 12 participants classified as having impaired cognitive function. The Holey Moley game scores ranged from 7 to 113. The socio-demographic characteristics of participants and game scores are listed in Table 3.

Table 3 : Socio-demographic characteristics and game scores

Item	Descriptive Value	Game Score Mean (SD)	Stat (p-value)
Age, year (SD)	47.1 (15.2)		(<0.01)[a]
Sex, n (%)			
Male	22 (46.8)	78.3 (25.7)	(0.493)[b]
Female	25 (53.2)	72.2 (33.)	
Race, n (%)			
Malay	19 (40.4)	67.4 (31.4)	(0.170)[c]
Chinese	19 (40.4)	75.4 (32.1)	
Indian	4 (19.1)	90.4 (15.4)	
Education Level, n (%)			
No formal education	1 (2.1)	50.0	(<0.01)[c]
Primary School	11 (23.4)	39.6 (33.3)	
Secondary School	21 (44.7)	80.4 (19.2)	
Diploma	13(27.7)	96.6 (11.4)	(<0.01)[d]
Post-graduation	1 (2.1)	98.0	
Marital Status, n (%)			
Single	8 (17)	79.3 (23.9)	(0.276)[c]
Married	38 (80.9)	75.4 (30.9)	
Divorced	1 (2.1)	28	
Co-morbidities, Mean of n comorbidities (SD)	1.1 (1.1)		(0.345)[a]

[a]= Pearson's correlation, [b] =Independent t-test, [c] = One-way ANOVA, [d]= Spearman's correlation

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There was a significant negative correlation between age and game score (r = -0.43a, p<0.01) indicating that with increasing age, game scores decreased. Additionally, there were no significant differences in game score between sexes. The majority of participants were Malay (n = 19), and Chinese (n = 19), and the remaining participants were Indian (n = 9). However, no significant differences in mean game score were found among races. Participants represented a variety of education levels (No formal education = 1, Primary School = 11, Secondary School = 21, Diploma = 13, Post-graduation = 1). There was a significant correlation between game score and level of education (r= 0.674, p<0.01). Number of comorbidities varied among participants (range 0-4), and there was a negative correlation between comorbidity and game score (r = -0.141, p =0.345).

Predicting cognitive status using mobile game score

The Holey Moley game was then validated against the MMSE, which is a gold standard for cognitive assessment. The game cut-off score for cognitive impairment was 63, which was determined by ROC curve (figure 1). A sensitivity and specificity test was performed to ensure reliability as shown in table 4. Validation and optimization of the Holey Moley game in predicting cognitive impairment is summarized

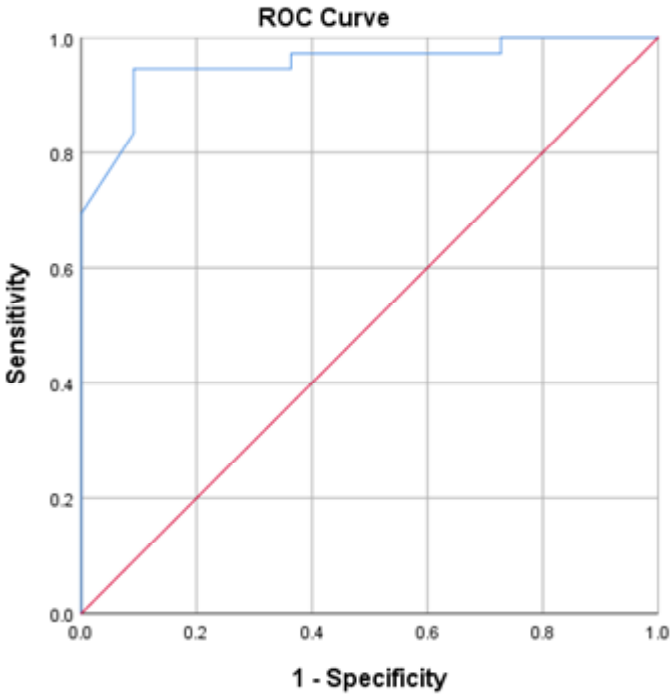

Fig. 1 : Area under the ROC curve for the validation of game scores

Table 4 : Mobile game and MMSE sensitivity and specificity cross tabulation

Mobile Game and MMSE Cross tabulation

			MMSE		Total
			Cognitive impairment	Normal cognition	
Mobile Game	Cognitive impairment	Count	11	1	12
		% within MMSE	100%	2.8%	25.5%
	Normal cognition	Count	0	35	35
		% within MMSE	0%	97.2%	74.5%
Total		Count	11	36	47
		% within MMSE	100%	100%	100%

Table 5 : Validation and optimization of the Holey Moley game in predicting cognitive impairment

Model	**n**	**Trade-off value**	**Sensitivity (%)**	**Specificity (%)**	**AUC ROC**	**95% CI**		***P*-value**
Validation	47	63.5	100	97.2%	0.953	0.894	1.0	<0.01

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The RMSE between the actual MMSE scores administered by physicians and the estimated scores based on mobile game was 1.8. The normalised RMSE was computed by dividing the RMSE by the value range of actual MMSE score (i.e., 30-9+1 = 22), was 8.1 %, showing that the proposed system could yield an accurate evaluation of MMSE (33). There was a significant positive correlation between MMSE and game scores (Pearson's correlation r= 0.92, P <0.01) (Figure 2). This indicates that our game-specific variables can capture the varying degrees of cognitive functions measured by the MMSE. Based on MMSE scores, there were 12 participants with impaired cognitive function. MMSE scores ranged from 9 to 30.

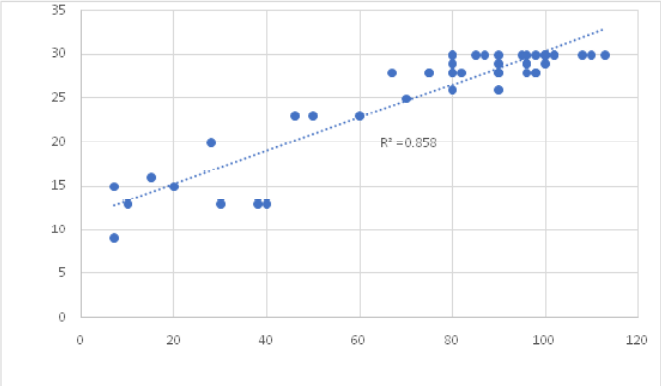

Fig. 2 : Correlation between MMSE scores and game scores

The goal of this study was to demonstrate the feasibility of a game-based cognitive assessment delivered on tablet technology that can be self-administered without the supervision of trained staff against standard mental status assessment tools. The Holey Moley game was selected based on its quick and user-friendly interface, time efficiency (60 seconds), and cost effectiveness. It was then validated against the MMSE.

Our findings showed that there was a significant negative correlation between age and game score, indicating that with increasing age, game score decreased. These findings are similar to a study which reported that, in general, older adults were less likely to use technology than younger adults. The relationship between age and adoption of technology was mediated by cognitive abilities, computer self-efficacy, and computer anxiety (34). This could be due to physiological decline in cognitive function, or lack of interest in mobile games among elderly participants.

In this study, participants came from different educational backgrounds, and game score correlated significantly with level of education. Participants with low levels of education performed poorly on the game despite having good cognitive status as assessed by MMSE. These findings demonstrate that patient characteristics, such as younger age and good educational background, are able to be tested using mobile devices. Number of comorbidities varied among patients (range 0-4), and there was a significant negative correlation between comorbidities and game score. It is already proven that physiological properties of comorbidities can reduce one's cognitive function (35). This reflects that patient's comorbidities can impair cognitive function.

The correlation of the Holey Moley game score with existing methods of clinical cognitive assessment (i.e. MMSE) is strong and may be useful in the detection of cognitive impairment. High R2, eg R2 > 0.6 and RMSE less than 10% ensures the model fits the data well. Thus, game-based assessment is a promising instrument for cognitive screening in clinical settings after proper validation. Our findings demonstrate that games can potentially revolutionise cognitive assessment in clinical settings, allowing for more frequent, affordable, and enjoyable assessments. Ideally, a suitably modified mobile game would be able to detect risk of cognitive impairment, and disease-related deterioration. Since the game-based assessment can be delivered independently, patients may be able to self-monitor. The game performance provided to healthcare providers may lead to appropriate interventions and/or investigations to ensure optimal treatment care.

Limitations

Further research is needed to generalise these results to different clinical conditions and settings. The design of this study was cross-sectional, each participant was only studied during their clinic visit, and played the game only once. Future research may assess the reliability of the game when played repeatedly by the same patient in clinic during follow-up to investigate the effects of prior exposure.

4. Conclusion

The freely available Holey Moley game is a promising instrument for cognitive screening in clinical settings. This work demonstrates the validity of games for cognitive screening that can be self-administered with minimal supervision from trained staff.

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Risk Assessment of Sleep Apnoea and Quality Of Sleep Among General Public in Klang Valley

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Abstract

Untreated Obstructive Sleep Apnoea (OSA) OSA can lead to various health complications and increase the risk of an automotive accident. It is believed that there is a significant correlation between OSA and quality of sleep. individuals with OSA should be diagnosed and treated as early as possible to improve their quality of life

Thus, this study aimed to identify the level of risk of OSA and quality of sleep among the general public in Klang Valley, Malaysia and to find out the association, difference and correlation between them. A cross-sectional study was carried out among 420 respondents who were recruited through convenience sampling from shopping malls in Klang Valley, Malaysia.Participants aged 18 years and above who agreed to participate in the survey were included in the study. Self-administered established questionnaires were used to assessOSA and Sleep quality. Data were analyzed using descriptive and inferential analysis.Mean (SD) age of the respondents was 38.52 (14.19) and 40% of them aged between 30 and 49. The majority of the study population were at low risk of having OSA (81.7%) and were poor sleepers (65.5%). It was found that gender, age categories, BMI categories, education level, employment status, co-morbidities and smoking were significantly associated with OSA ($p<0.05$) and there was a significant difference in the mean global PSQI score ($p<0.05$) between the races, age categories, and religions. The risk of OSA was significantly associated with quality of sleep ($p=0.011$, contingency coefficient=0.123) where poor sleepers (21.8%) were at higher risk of OSA. OSA was significantly correlated with the quality of sleep ($r=0.124$, $p=0.011$).The majority of the study population was at a low risk of OSA. However, individuals at high-risk OSA were found to have poor sleep quality. Therefore, OSA may develop in poor sleepers over a period of time. Study findings will help healthcare providers and policymakers to educate and spread awareness about OSA among the public. This will be beneficial in the early diagnosis and treatment of OSA before it complicates to other co-morbidities.

Key words : Obstructive sleep apnoea (OSA); Quality of sleep; Berlin questionnaire; PSQI Questionnaire; Malaysia.

1. Introduction

Obstructive Sleep Apnoea (OSA) is a common sleep-related breathing disorder, characterizedby a repetitive episode of partial or complete upper airway obstruction during sleep, despite the persistent effort to breath (1,2). World Health Organization (WHO) declared that OSA has influenced more than 100 million people worldwide and is reported to be highly prevalent (3). The prevalence of OSA is reported from as low as 15% in Singapore to as much as 78% in Brazil (3,4). The prevalence of OSAin community-dwelling adults aged between 30 - 70 years in Malaysia is determined to be 8.8% and 5.1% in males and females respectively. These results obtained in Malaysia are almost twice as compared to those reported by Young et al.(5).

OSA is typically asymptomatic in its initial stages;therefore, the patients remain unaware unless noticed by the family members (6). The actual signs and symptoms appear in the later course of the disease when abnormal breathing pattern is observed by the family members and suspected by the physician on routine check-up (7). The substantial signs and symptoms of OSA include heavy snoring, frequent nocturnal awakenings leading to fragmented sleep, impaired quality of sleep triggering excessive daytime sleepiness (2,8). Prolonged untreated OSA is associated with significant morbidity comprising cardiovascular, metabolic, and neurocognitive

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complications (2,8) and may result in impaired working performance and a higher risk of road accidents (9).

An individual's Quality of Life (QOL) is highly dependent on the quality and duration of sleep, therefore, changes in quality of sleep may affect an individual's QOL (10). OSA is one of the risk factors that may contribute to poor quality of sleep. Individuals with OSA should, therefore, be diagnosed and treated as early as possible to improve their quality of life and prevent the occurrence of various complications and risks for a road traffic accident.

Few studies have been carried out in Malaysia, reporting the prevalence of OSA among community-dwelling adults and bus drivers (11,12) and quality of sleep among medical students (13). None of the studiesdetermined the association of OSA and quality of sleep in Malaysia. Therefore, this study was aimed to determine the risk of sleep apnea, quality of sleep and evaluate the association between them.

2. Materials and Methods

Study design and setting

A cross-sectional survey was done on 420 participants. They were recruited conveniently from different shopping malls in KlangValley, Malaysia. Those participants who were Malaysian, 18 years or older and agreed to participate in the survey were included.

Sample size

According to the Department of Statistics Malaysia, the population size of Klang Valley, Malaysia is approximately 7.9 million. The total sample size for this study was 385, and it was calculated using Sample Size Calculator by Raosoft®, Inc. with a margin of error of 5%, 95% confidence interval and response distribution of 50%(14).A total of 420 respondents were chosen, approached individually and enrolled in this study.

Survey items

A pre-validated, self-administered questionnaire was adapted from previous studies and translated into BahasaMelayu and Chinese by a certified translating agency, MSB Venture (SA0352850-U). The questionnaires comprised of three main sections. Section A captured the basic socio-demographic data of the respondents. Section B consisted of the Berlin Questionnaire to assess the risk of having OSA(15). It consists of ten closed-ended questions, and they are categorizedinto three categories according to the factors evaluated, such as snoring frequency and severity, the severity of daytime sleepiness and history of high blood pressure and obesity Respondents are considered having a high risk of OSA if they scored positive for two or more categories and low risk if they scored positive for one or none of the categories among the three and Section C consisted of Pittsburgh Sleep Quality Index (PSQI) questionnaire to assess the quality of sleep of the respondents(16).It consists of nineteen self-rated questions used for PSQI scoring. These nineteen self-rated questions are combined to yield seven component scores;each of them ranges between zero to three points.All the seven component scores are then added up to obtain the global PSQI score, which ranges between 0 to 21 points. For the results, the global PSQI score that is lower than five indicates that the respondent is a good sleeper while greater than five indicates that the respondent is a poor sleeper. Higher points indicate the worse quality of sleep experienced by the respondent. Permission to use Berlin and PSQI questionnaires were taken from both the authors.

Data collection and ethics

Respondents were explained about the purpose of this study and written informed consent was obtained before the survey begins. Ethical approval was taken from the Research Management Centre (RMC), MAHSA University. Patients who refused to participate in this study were excluded.

Data analysis

Statistical analyses were performed using the IBM SPSS Statistics Version 23. The data collected were expressed as descriptive statistics such as frequencies, percentages, mean as well as inferential statistics. Categorical data were expressed as proportions, n (%) whereas continuous data were expressed as means ± standard deviation (SD). The normality of the data was checked and determined by using the normality test. Since the data obtained was normally distributed, parametric tests were used for data analysis. The tests employed were independent sample t-test, one-way ANOVA and Pearson's correlation.

The association between the risk of having OSA and categorical data was evaluated using the chi-square test and differences with continuous variables were estimated using independent t-test. Besides, the differences between global PSQI score (continuous variable) and socio-demographic data wereassessed using independent t-test and one-way ANOVA, whereas the correlation between them wasexamined using Pearson's correlation.

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3. Results and Discussion

Pilot study

To check the clarity and appropriateness of the translated questionnaire, the Malay version of the Pittsburgh Sleep Quality Index (PSQI-M) and Berlin Questionnaire (BQ-M) was pre-tested in a pilot study of 30 individuals. The internal consistency of the translated PSQI-M in the pilot study, measured by Cronbach's alpha, was 0.68 while Cronbach's alpha value for the Category 1 and 2 of BQ-M was 0.76 and 0.86 respectively.

Socio-demographic data of the respondents

Out of 450, 420 participants gave their consent and voluntarily enrolled in this survey and return the questionnaire to the principalinvestigator;therefore, the response rate of this study was 93% (420/450) that surpassed the good index of response rate. It can be observed that most of the respondents who enrolled in this study were within the mean age 38.5 (+14.1)and the majority of them were female (59%) and Malay (43.1%). Most of the respondents were non-smoker (91%), non-alcoholic (81.7%) with no co-morbidities (78.3%).

Risk of obstructive sleep apnoea (OSA) and Quality of sleep

The result of berlin questionnaireshows that 18.3% (n=77) of the general public was at high-risk for OSA (Figure 1). While results from PSQI, 65% (n=275) was identified as poor sleepers (Figure 2).

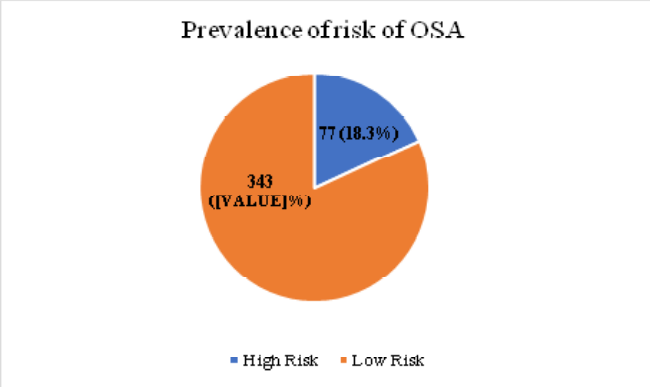

Figure 1 : Prevalence of risk of OSA

Association and differences of risk of obstructive sleep apnoea (OSA) across the various socio-demographic characteristics of the respondents

It has been observed that there were statically significant association between gender (p=0.001), age categories(p=0.001), BMI categories (p<0.001), education levels (p=0.015), employment status (p=0.013), co-morbidities (p <0.001), smoking (p=0.042)with risk

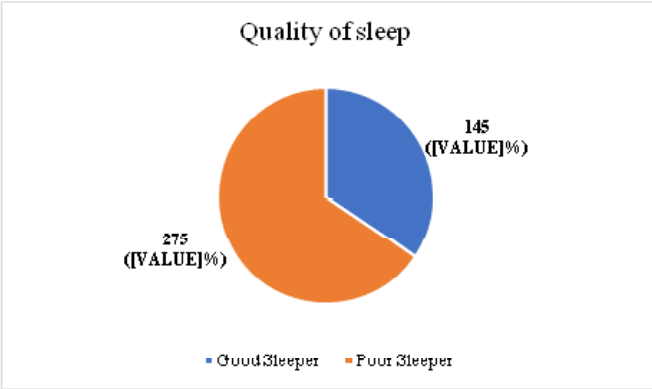

Figure 2: Results of Pittsburgh Quality of Sleep (PSQI)

of having OSA (Table 1). Significant differences were reported for mean age (p <0.001) and mean BMI (p <0.001) between low and high-risk OSA groups. It was reported that individuals with greater mean age (44.31±15.05 years) and greater mean BMI (29.44±5.31 kg/m2) were at a higher risk of having OSA.

Score of Pittsburgh Sleep Quality Index (PSQI)

The PSQI score ranged from 0 (minimum) to 15 (maximum) with a mean score of 5.56 ± 2.80. Out of 420, 65.5% (n=275) of the respondents achieved the score 5 and more than 5, indicates poor quality of sleep while 34.5% (n=145) attained the score less than 5 which suggestive of good quality of sleep (Table 2).

Table 2 : Scores of Pittsburgh Sleep Quality Index

Pittsburgh Sleep Quality Index	Score	Frequency (%)
Total PSQI score		
Minimum	0	
Maximum	18	
Mean (SD)	5.86 ± 2.80	
Good	< 5	145 (34.5)
Poor	≥ 5	275 (65.5)

Differences between the socio-demographic factors and PSQI score

There were significant differences in the mean PSQI score between age categories (p=0.038), race (p=<0.001), and religion (p=0.017) as shown in Table 3.

Correlation between sleep quality with age and BMI

Regarding the correlation between the quality of sleep with age and BMI, there was a significant weak negative correlation between the age and mean global PSQI score (r= -0.113, p=0.016) (Table 4).

OSA risk and quality of sleep

On univariable analysis to determine the association

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Table 1: Association and difference between risk of OSA across the various socio-demographic characteristics of the respondents (n=420)

Variables		**Risk of having OSA**		***p*-value**
		Low risk	**High risk**	
Gender Male Female	n (%)	 127 (73.8) 216 (87.1)	 45 (26.2) 32 (12.9)	**0.001**[a]
Age	Mean (±SD)	37.22 (±13.68)	44.31 (±15.05)	**<0.001**[b]
Age categories (years) 18-29 30-49 50-64 >65	n (%)	 126 (87.5) 141 (83.9) 64 (73.6) 12 (57.1)	 18 (12.5) 27 (16.1) 23 (26.4) 9 (42.9)	**0.001**[a]
BMI (kg/m^2)	Mean (±SD)	24.11 (±4.13)	29.44 (±5.31)	**<0.001**[b]
BMI categories Underweight Normal Overweight Obese	n (%)	 21 (100.0) 185 (92.0) 103 (82.4) 26 (41.3)	 0 (0.0) 16 (8.0) 22 (17.6) 37 (58.7)	**<0.001**[a]
Race Malay Chinese Indian Kadazan	n (%)	 153 (84.5) 145 (81.5) 44 (73.3) 1 (100.0)	 28 (15.5) 33 (18.5) 16 (26.7) 0 (0.0)	0.261[a]
Religion Muslim Hindu Christian Buddhist Sikh Freethinker	n (%)	 154 (85.1) 35 (72.9) 28 (73.7) 122 (82.4) 1 (100.0) 3 (75.0)	 27 (14.9) 13 (27.1) 10 (26.3) 26 (17.6) 0 (0.0) 1 (25.0)	0.318[a]
Marital status Single Married Divorced	n (%)	 151 (86.3) 187 (77.9) 5 (100.0)	 24 (13.7) 53 (22.1) 0 (0.0)	0.053[a]
Education No formal education Primary school Secondary school Diploma Bachelors Masters PhD	n (%)	 1 (100.0) 8 (61.5) 82 (75.2) 81 (77.1) 142 (88.2) 26 (92.9) 3 (100.0)	 0 (0.0) 5 (38.5) 27 (24.8) 24 (22.9) 19 (11.8) 2 (7.1) 0 (0.0)	**0.015**[a]
Employment status Employed Unemployed Retired Housewife Student Own business	n (%)	 226 (81.0) 16 (88.9) 18 (62.1) 24 (80.0) 52 (94.5) 7 (77.8)	 53 (19.0) 2 (11.1) 11 (37.9) 6 (20.0) 3 (5.5) 2 (22.2)	**0.013**[a]

[a] = Chi-square (p-value <0.05 = significant association (p-value <0.05 = significant difference) OSA: Obstructive Sleep Apnoea

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Table 3 : Differences between the socio-demographic characteristics and PSQI score

Variables	**PSQI Score**		
	Mean (±SD)	**95% CI**	***p*-value**
Gender			0.449[a]
Male	5.78 (2.89)	-0.68 – 0.40	
Female	5.92 (2.75)		
Age category (years)			**0.038[c]**
18 – 29	6.41 (2.76)	5.96 – 6.86	
30 – 49	5.63 (3.04)	5.16 – 6.09	
50 – 64	5.48 (2.31)	4.99 – 5.98	
> 65	5.67 (2.59)	4.49 – 6.85	
BMI category			0.881[c]
Underweight	6.00 (2.28)	4.96 – 7.04	
Normal	5.87 (2.99)	5.45 – 6.28	
Overweight	5.73 (2.74)	5.24 – 6.21	
Obese	6.06 (2.42)	5.46 – 6.67	
Race			**<0.001[c]**
Malay	6.30 (2.91)	5.88 – 6.73	
Chinese	5.20 (2.51)	4.83 – 5.57	
Indian	6.55 (2.93)	5.79 – 7.31	
Kadazan	4.00 (-)	-	
Religion			**0.017[c]**
Muslim	6.28 (2.92)	5.85 – 6.71	
Hindu	6.23 (3.05)	5.34 – 7.12	
Christian	6.05 (2.25)	5.31 – 6.79	
Buddhist	5.22 (2.64)	4.79 – 5.64	
Sikh	7.00 (-)	2.03 – 7.47	
Freethinker			
Marital status			0.060[c]
Single	6.23 (2.89)	5.80 – 6.66	
Married	5.59 (2.70)	5.25 – 5.93	
Divorced	6.60 (3.78)	1.90 – 11.30	
Employment status			0.133[c]
Employed	5.70 (2.86)	5.36 – 6.04	
Unemployed	6.28 (1.93)	5.32 – 7.24	
Retired	5.21 (2.21)	4.37 – 6.05	
Housewife	6.20 (2.46)	5.28 – 7.12	
Student	6.71 (2.98)	5.90 – 7.51	
Own business	6.11 (3.48)	3.44 – 8.79	
Gross monthly income			0.191[c]
< RM 1000	6.32 (2.76)	5.85 – 6.79	
RM 1000 – 2999	5.81 (3.02)	5.17 – 6.46	
RM 3000 – 4999	5.60 (2.91)	5.04 – 6.16	
RM 5000 – 6999	5.89 (2.68)	5.15 – 6.63	
RM 7000 – 8999	5.06 (2.21)	3.96 – 6.15	
RM 9000 – 10,000	4.25 (1.98)	2.59 – 5.91	
> RM 10,000	5.79 (2.15)	4.54 – 7.03	

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Co-morbidities			0.384[a]
	6.08 (2.87)	-0.36 – 0.93	
Yes	5.79 (2.75)		
No			
Smoking			0.976[c]
	5.91 (2.79)	5.63 – 6.19	
Non-smoker	5.07 (2.55)	4.08 – 6.06	
Light smoker	5.78 (3.23)	3.29 – 8.26	
Moderate smoker	5.00 (-)		
Heavy smoker		-	
Alcohol intake			0.063[c]
	6.03 (2.85)	5.73 – 6.33	
Non-alcoholics	5.29 (2.23)	4.76 – 5.83	
Social drinker	3.13 (1.96)	1.49 – 4.76	
Moderate drinker			
Heavy drinker	4.00 (-)	-	

[a] = Independent t-test (p-value <0.05 = significant difference); [b] = Pearson's Correlation (p-value <0.05 = significant difference); [c] = One-way ANOVA (p-value <0.05 = significant difference); CI= Confidence Interval

Table 4 : Correlation between sleep quality with age and BMI

Age	r_s= -0.113	**0.021**[b]
BMI	r_s= -0.018	0.710[b]

between quality of sleep with risk of OSA. Good sleepers (88.3%) had a low risk of OSA as compared to poor sleepers (78.2%)(Table 5). Similarly, poor sleepers were at higher risk of having OSA (21.8%)(p=0.011). This indicated that there was a significant positive correlation between the variables (r=0.124, p=0.011). The correlation between OSA risk and quality of sleep was expressed using Phi and Cramer's V value. Both the values were found to be the same, which was 0.124.

Table 5 : Association between OSA risk with quality of sleep

Quality of sleep		**Good sleepers (<5)**	**Poor sleepers**	***p*-value**
		n (%)	**(>5)**	
OSA risk			**n (%)**	
Low risk	n (%)	128 (88.3)	215 (78.2)	**0.011**[a]
High risk	n (%)	17 (11.7)	60 (21.8)	

[a] = Chi-square (p-value <0.05 = significant association)

The current study aimed to identify the risk of OSA and quality of sleep through validated research tools among the general public. Our result showed that 18.3% and 65.5% of enrolled respondents had a high risk of OSA and poor sleep quality, respectively.

Our study revealed a significant association betweenthe risk of having OSA among males (26.2%) as compared to females (12.9%). This finding is consistent with several studies supporting that the male gender is significantly associated with developing the risk of OSA(1,2,17).A study from Iran reported that ;male respondents had a higher risk of OSA (51.4%) as compared to females (26.5%)(18). Similarly, males showed higher prevalence of OSA (12.6%, n = 95) as compared to females (3.3%, n = 27) in a recent North West Adelaide Health Study (NWAHS), 2018 (19).

Age has a linear relationship with OSA risk (20-22); elderly tends to have a higher risk of OSA because of reduced respiratory efficiencydue to the aging processwhich increases the prevalence of OSA in the older population(23).Jordan et al. proposed that the upper airway dilator muscles in older individuals might not work as efficiently as in the younger (2). The aged population might experience airway collapse easily due to loss of collagen or might arouse easily due to ,more mediocre quality of sleep (2).

Several studies have reported that obesity is one of the main risk factors that contributes in the development of OSA (1,2,17,18). Obese participants (58.7%) were at higher risk of having OSA as compared to non-obese in the present study. In a Wisconsin cohort study with a 4-year follow-up, the risk of developing OSA among those who do not have OSA at the beginning was six-fold higher as the weight increases by 10%(23). Excessive fats deposition at the neck region could increase the likelihood of airway obstruction, especially during supine sleep position with the pull of gravity(2).

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The level of education is also significantly associated with developing the risk of OSA. Foroughi et al. reported that respondents with a low educational background were at higher risk of OSA (18). Likewise,Sunwoo et al. also recorded high OSA risk among the respondents with a middle or low level of education (OR=1.60; 95% CI, 1.24 - 2.07), (24). Individuals with a higher level of education are more concerned about their health as compared to those with a lower level of education (25).

Individuals with self-reported co-morbidities (42.9%) were significantly at higher risk of having OSA as compared to those without co-morbidities (11.6%) (p=<0.001). Mild (56.2%), moderate (67.6%) and severe OSA (70.0%) OSA was observed in people with co-morbid conditions by Pinto et al in 2016.Tveit et al. also reported that the prevalence of some cardio-metabolic diseases (e.g. hypertension, diabetes mellitus, obesity) were higher with greater severity of OSA(27). Higher mortality risk was identified among OSA patients with comorbidities as compared to those without co-morbid condition in Taiwan by Chiang et al(HR: 11.01, 95% CI 4.00-30.33, p = <0.01) (28). Evidence suggests that co-morbidities might cause changes in the physiological functions of the body systems and subsequently leads to OSA.

Smoking had also shown as one of the risk factors of developing the OSA. Smoking caused sleep disturbance, nicotine-related relaxation of the upper airway, and inflammation in the upper airway because of inhalation of smoke (2,31) Our results depicted risk of OSA was significantly associated with smoking. Active smokers had a high OSA risk than non-active smokers in Nigeria (OR=28.67, 95% CI, 1.43 - 576.31, p=0.028) (29). According to the Wisconsin cohort study, there wasthree times higher risk of developing OSA in current smokers as compared to the former or never smokers (30).

Majority of our study participants had a poor quality of sleep which is consistent with the previous literature (32). Quality sleep is essentialfor all age groups, regardless of ethnicity. Poor quality sleep was significantly prevalent among Indians in the present study. According to NHMS 2014, Indians were highly prevalent (28.1%) to be obese, which is also one of the risk factors of OSA as discussed above, followed by Malays (22.0%) (33).

Younger adults aged 18 - 29 were having the highest mean PSQI score as compared to the other age categories. The younger generation nowadays is addicted to video games, which may lead to irregular sleep timings. According to the statistics in Malaysia, 28.0% of the gamers were maleaged between 21 - 35(34).Peracchia et al. concluded that exposure to video games for a long period, especially in the evening, can significantly cause sleep problems to arise and subsequently leads to poor quality of sleep(35).Correlation analysis showed a weak negative association of age with mean global PSQI score (r= -0.113, p=0.021).Therefore, the younger the age of an individual, the higher the mean global PSQI score would be, and thus more inferior their quality of sleep.

Moreover, young adults aged 18 - 29 were mostly students in tertiary educational institutes or fresh graduates,who are at the beginning of their careers and professional life. Poor sleep quality among such a group of people might be due to work stress or vice versa, as indicated by Valerio et al. (p=<0.001)(36).

There was a significant difference in the mean PSQI score between different religions. Muslims had a higher mean PSQI score (6.28±2.92) as compared to the non-Muslims. Prayer time was one of the factors affecting the Muslim's sleep schedule(37). According to the religious practice of Muslims, praying five times a day, starting with the first prayer (Fajr) during the dawn, which is roughly estimated to be one or one and half hours before sunrise(38). Hence, Muslims might be experiencing a shorter duration of sleep as compared to individuals of other religions due to the early rise for prayers, specifically those who went to bed late.

Univariate analysis showed that respondents with good quality of sleep had a low risk of OSA and vice versa, which is in line with Sokwalla et al.study results that concluded a significant association between the risk of OSA and poor quality of sleep among individuals from Kenya. Good sleepers (70.2%) had lower risk of OSA as compared to the poor sleepers (43.9%)(39) and OSA affects the quality of sleep negatively(40).

Limitations

Few limitations should be considered when interpreting the results from this study. Given that our study is a cross-sectional study design, so the results can't be generalized and represent all state of Malaysia since the study was conducted in 12 selected shopping malls in different areas of KlangValley. Other limitations included the time and budget constraints. Besides, the findings of this study may be biased in terms of memory and information because the data obtained such as the subjective sleep quality and the major variables were self-

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reported. Thus, it might differ from the actual situation. Other than that, the willingness of the respondents to provide information in some short open-ended questions and the accuracy of the data should be considered because they might potentially affect the study results. Last but not least, sleep measurement was based on subjective descriptions rather than objective assessment. There might be a memory gap between certain variables such as total sleep time and sleep onset, subsequently influencing the classification of people with good or poor sleep quality based on the total score of PSQI and the results of this study.

4. Conclusion

The majority of the study population was at low risk of OSA, even though most of them were poor sleepers. However, high-risk OSA individuals were found to have poor sleep quality. Therefore, OSA may develop in poor sleepers over some time. Study findings will help healthcare providers, community pharmacists and policymakers to educate and spread awareness about OSA and quality of sleep among the general public. Early diagnosis and treatment of OSA,and managing the quality of sleep before it complicates to other co-morbid conditions is deemed necessary.

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Conflict of Interest

The authors declared no conflict ofinterest.

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Anti-Angiogenic Effect of Ethanolic Extract and its Phenolic Rich Fraction of *Acacia auriculiformis* Bark in the Chick Embryo Chorioallantoic Membrane Model

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Abstract

Acacia auriculiformisplant is widely used in traditional medicines for treatment of various diseases. The main objective of this study is to evaluate the anti-angiogenic effect of ethanolic extract and its phenolic rich fraction of A. auriculiformis bark in the chick embryo chorioallantoic membrane model. Dried powdered bark of A. auriculiformis was extracted with 70% ethanol and the resultant was partitioned with hexane, ethyl acetate and aqueous. Folin-Ciocalteu assay was used to quantify the phenolic content in fractions of A. auriculiformis bark. The anti-angiogenic effect of ethanolic extract and phenolic rich fraction were evaluated by using in-ovo chorioallantoic membrane (CAM) model. The reduction in total blood vessels number in the CAM model was considered as positive indicator of anti-angiogenic effect. Ethyl acetate fraction showed the highest phenolic content which was 621±16.20 mg of gallic acid equivalent per gram of fraction. CAM treated with ethanolic extract (250 μg, 500 μg), ethyl acetate fraction (10 μg & 50 μg) and prednisone (250 μg) showed significant reduction ($p<0.05$) in total blood vessel (TBV) 46.4±0.89, 36.4±2.30, 47.6±3.05, 37.6±1.82 & 37.0±2.00 compared with negative control group (61.7±2.52). The anti-angiogenic effect shown by the ethanolic extract and ethyl acetate fraction of A. auriculiformis might be due to the presence of phenolic compound. A. auriculiformis can be a new source of anti-angiogenic agent in anticancer therapy.

Key words : Anti-angiogenesis, A. auriculiformis, ethanolic extract, phenolic rich fraction, CAM assay

1. Introduction

Angiogenesis is the development of new blood vessel from pre-existing capillaries.Angiogenesis is involved in tissue repair and granulation tissue formation. In healthy adults, angiogenesis takes place during tissue repair and the female reproductive cycle(1). Angiogenesis is essential for tumour growth and metastasis. Folkman(1) observed the limited development of tumour with no blood vessels. The maximum diameter of avascular tumour are less than 1mm(1). Cancer cells are unable to get into the circulation before the development of blood vessel on the tumour. Vascularized tumor has a higher probability of metastasizing than prevascular tumor(2).Angiogenic activators promote angiogenesis and angiogenic inhibitors inhibit angiogenesis. Examples of angiogenic activators are vascular endothelial growth factor A (VEGFA), fibroblast growth factors (FGFs), platelet-derived growth factor (PDGF) and epidermal growth factor (EGF). Examples of angiogenic inhibitors are thrombospondin 1, angiostatin, endostatin and tumstatin. Development of new vessels are controlled by a balanced mixture of angiogenic activators and angiogenic inhibitors(1).

Acacia auriculiformis belongs to the family Fabaceae.A. auriculiformis is a multipurpose leguminous tree that is important in medicinal and forestry fields. Many therapeutically active constituents are derived from this plant(3).A. auriculiformis is used traditionally as antimalarial medication and to treat eyes conditions & skin diseases(4).A. auriculiformis contains carbohydrates, tannins, anthocyanidins, flavonoids and saponins(3).A. auriculiformis has a unique saponin due to presence of tridesmoside saponins. The saponins are Proacaciaside-I, Proacaciaside-II, acaciamine, Acaciaside A & Acaciaside B. Acaciaside A &Acaciaside B are proved to be responsible for the antifilarial, antimicrobial, spermicidal activities(3).Acacia auriculiformis is chosen as a potential candidate to provide phytoconstituents with antioxidant activity. From the current studies, the bark of A. auriculiformis is known to have the antioxidant activity due to itsphenolic content(5). The extract from the bark has showed quenching capacity on DPPH, ABTS, OH-, O2- and NO(3).A. auriculiformis is a potential source of antioxidants which could be useful as pharmaceutical products(6).

The main objective of this study is to evaluate the anti-angiogenic effect of ethanolic extract and phenolic rich

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fraction of A. auriculiformis bark in the chick embryo chorioallantoic membrane model.

2. Materials and Methods

Collection and authentication of plant materials

The barks of Acacia auriculiformis were collected inCheras, Malaysia. Authentication of plant (UPM/IBS/UB/H23/19)was obtained from Dr. Mohd Hafizi Adzmi Hanafi from the Biodiversity unit of University Putra Malaysia (UPM).

Preparation of crude extract

Fresh bark was collected and cleaned with water, dried in dark and dust-free environment. The dried bark was then powdered using a blender and 70% ethanolic extract was prepared by maceration method(7). About 100g of powered dry bark was soaked with 2 liter of 70% ethanol and the mixture was stirred twice daily for 4 days. The extract was filtered through the filter paper and the excess solvent was removed by using rotary evaporator. Finally, supernatant solution was lyophilized and dried crude extract was kept in airtight container(7).

Preparation of phenolic rich fraction (PRF)

5g dried ethanolic extract was dissolved in 100ml of water and sequentially fractionated in a separatory funnel with 100 ml hexane and 100 ml ethyl acetate. The solvent in these fractions was evaporated by the rotavapor to prepare the dried fractions. Extract and fractions were dissolved with 70% ethanol todetermine the phenolic content(7).

Folin-Ciocalteu assay

Folin-Ciocalteu method was used to determine the total phenolic content (TPC) of the above fractions. The concentration of phenolic content in each fractionwas derived from a gallic acid calibration curve. To prepare a calibration curve, 20, 40, 60, 80 and 100 micrograms of gallic acid were dissolved in 1ml of 70% ethanol and mixed with 5 mL of Folin-Ciocalteu reagent (10%v/v) respectively. These mixtures were incubated in the dark at room temperature for 3 minutes. Then, 3.0 g of anhydrous Na2CO3 in the form of 4?mL of Na2CO3 (7.5%w/v) was added to each mixture. These mixtures were further incubated in the dark for 90 minutes. Then, the absorbance was read at 765 nm using double beam UV spectrophotometer(8). The calibration curve was the graph of absorbance value against the respective concentration of gallic acid. The absorbance of the fractions was read using a similar procedure as descried for the gallic acid. All determinations were performed in triplicate(9).

The total phenolic content of fraction was expressed in gallic acid equivalents (GAE) using the formula(10):

$$A = \frac{(C)(V)}{m}$$

A = total phenolic content of fraction, mg/g plant extract in GAE

C =concentration of gallic acid established from the calibration curve (mg/ml)

V = volume of extract in millilitre

m = weight of dry plant extract in gram

Preparation of test samples

Negative control : 10 μL of phosphate buffer solution (PBS) /pellet

Positive control : 250μg prednisone/pellet

Extract : 250 μg/pellet and 500 μg/pellet

Phenolic rich fraction : 10 μg/pellet and 50 μg/pellet

Circular pellet were cut from a piece of filter paper with a paper puncher(11).Circular discs were sterilized by exposing them under UV light for at least 5 minutes. The pellet was infused with different solution and used as an implant. Prednisone, ethanolic extract & phenolic rich fraction were dissolved in PBS in different concentration. Then the solution was added drop wise on the pellet using the micropipette. The pellet infused with solution was lightly placed on the Chorioallantoic membrane (CAM) using sterile forceps.

Chorioallantoic membrane (CAM) assay

30 fertilized fresh eggs (within 7 days postlaying) were obtained from Lay Hong Sdn Bhd, Klang, Malaysia. These eggs were assigned into 6 treatment groups (n=5) for each treatment group. Eggshell surface was cleaned with moist tissue paper to remove any dirt. Then, eggshell surface was cleaned with 70% ethanol. Eggs were placed horizontally on suitable egg tray. No rotation of the eggs was required throughout the experiment(11). Eggs were incubated at 37-degreeCelsius and 60% humidity for 2 days.

On incubation day 3, 2-3ml of albumin was removed from each egg though the acute pole of the egg using 25G hypodermic needle and 3ml syringe. Removal of albumin dissociated the CAM from the eggshell membrane by creating a false air sac directly over the CAM.On incubation day 4, a square window (approximately 10 x 10 mm) was opened on the shell. A rotating carborundum disc was used to cut the shell using without damage the underlying eggshell membrane.

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Several drops of phosphate buffer solution were applied to moisten the eggshell membrane and wash away the shell dust. Forceps were used to remove whole piece of shell(12).The window was sealed with paper clinical tape and eggs were returned the into the incubator(13).On day 10 of incubation, implant was placed onto the CAM.CAMs were photographed with a camera after 24 hours. Total number of blood vessel was counted in an area of 3x3cm on the CAM membrane.

All the equipment, instrument and working area were cleaned with 70% ethanol before any procedure. Eggs were cleaned before incubation to remove egg outer surface debris(13). The making of square window on the eggshell and the placing of the implantation were performed in sterile condition (within the laminar flow hood in the clean room). All these precautions were to reduce the risk of contamination.

Percentage inhibition calculation

The total blood vessel (TBV) number was the sum of number of primary, secondary and tertiary blood vessels(14). The percentage inhibition was derived from total blood vessel number.

Calculation of percentage inhibition using the following formula (14) :

Percentage inhibition (%) = [(TBV of CAM treated by phosphate buffer- TBV of CAM treated by extracts/ fraction)/(TBV of CAM treated by phosphate buffer)] × 100%

Statistical analysis

All the values was recorded as mean ± standard error of mean (S.E.M.) the data was analyzed using one-way ANOVA with the differences of the means considered significant at $p<0.05$.

3. Results and Discussions

Folin-Ciocalteu assay

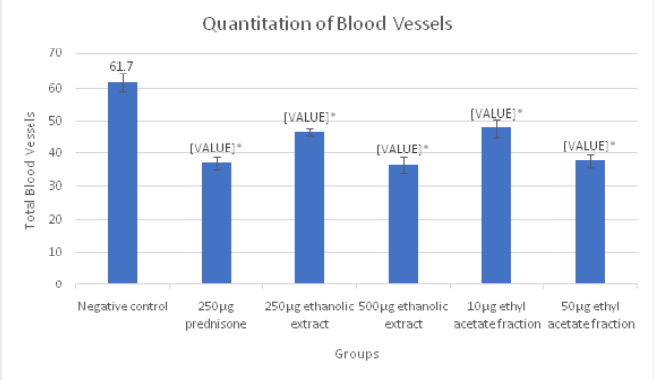
Total phenolic content in different fraction of A. auriculiformis was expressed as milligrams of gallic acid equivalents (GAE). Ethyl acetate fraction was the highest (621±16.20) followed by hexane fraction (398±2.89) and aqueous fraction (267±5.69) mg of gallic acid per gram of aqueous fraction as shown in Table 1.

Table 1: Total phenol content in mg of gallic acid equivalent per gram of each fraction

Fractions	Total phenol (mg gallic acid/g)
Aqueous	267±5.69
Hexane	398±2.89
Ethyl acetate	621±16.20

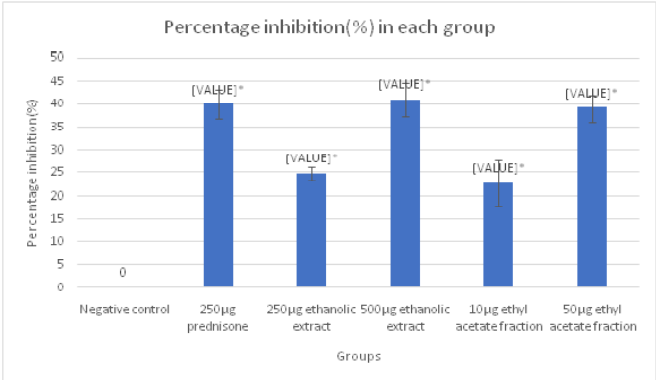
CAM assay

The total number of blood vesselsand percentage inhibition are used as the main criteria for analysis of anti-angiogenic effect. Reduction of blood vessel in test groups are significantly ($P< 0.05$) different compared to negative control group. The 500 µg of ethanolic extract showed most least number of TBV (36.4±2.30) compared with 250 µg of ethanolic extract (46.4±0.89) and 10 µg & 50 µg of ethyl acetate fraction (47.6±3.05, 37.6±1.82) (Graph 1 and 2). The reduction of blood vessels on the CAM confirmed the anti-angiogenic potential of the extract and fractions. Percent vascularity inhibition (PVI) was calculated to determine the degree of inhibition of blood vessel formation exerted by the A. auriculiformis extract and fraction.500µg of ethanolic extract treatment group showed highest PVI (41.0±3.73%) compared with 250 µg of ethanolic extract PVI (24.8±1.47) and 10 µg, 50 µg of ethyl acetate fraction PVI (22.8±4.94, 39.1±2.94) (Fig 1).



Graph 1 : Quantitation of blood vessels in treatment groups

* Significantly different at $P\leq0.05$ by Tukey's HSD test compared with negative control group.



Graph 2 : Percentage inhibition in treatment groups

* Significantly different at $P\leq0.05$ by Tukey's HSD test compared with negative control group.

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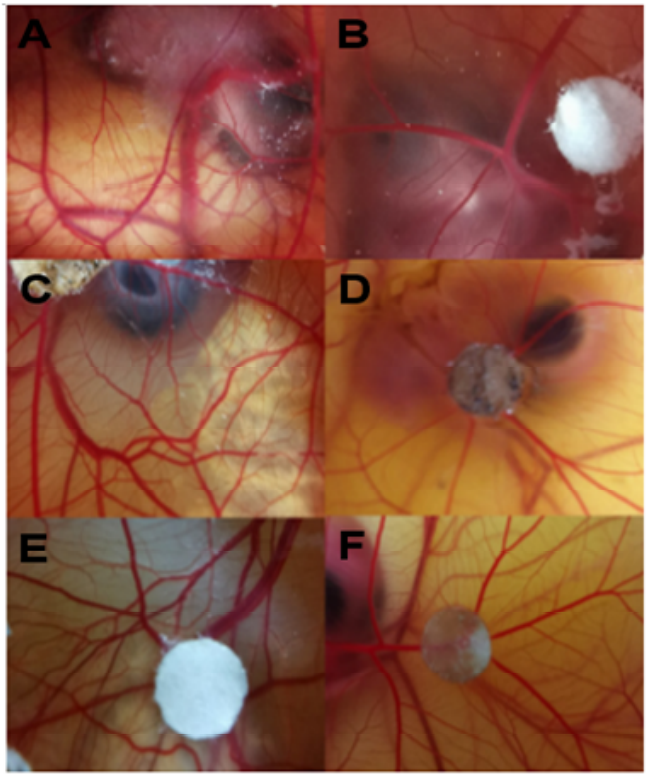

Fig 1: Representative image of CAM from each treatment groups.

CAM treated with negative control (A), 250µg of prednisone as positive control (B), 250µg of ethanolic extract (C), 500µg of ethanolic extract (D), 10µg of ethyl acetate fraction (E) & 50µg of ethyl acetate fraction (F).

Cancer causes a huge number of morbidity and mortality worldwide and is predicted to be responsible for 9.6 million deaths in 2018(15). Chemotherapy is the mainstream choice in management of a range of cancers. Chemotherapy also affects all rapidly dividing normal tissues(16). Chemotherapy can cause many side effect like bone marrow toxicity, neurotoxicity and cardiotoxicity(17, 18). Moreover, the cancer cells develop resistance to cytotoxic drugs by mutation during cytotoxic treatment. This mutation creates tumour cells that are less susceptible to the drugs(16).Epidemiological studies suggest diet with high number of antioxidants can reduce the risk of cancers significantly. The intake of dietary antioxidants has now received further attention because of universal acceptability based on its safety and less side effects potential(19).Various extracts of this plant have shown antioxidant benefit(6).

Folin-Ciocalteu assay was performed to quantify the phenolic content of the fractions of A. auriculiformis(8). Folin assay reveled that phenolic compound was present in different concentration in each fraction. The ethyl acetate fraction as the phenolic rich fraction was having the highest phenolic content which was equivalent to 621±16.20mg of gallic acid per gram of dried fraction powder. The ethyl acetate as the phenolic rich fraction was having the highest potential in showing the highest significant anti-angiogenesis activity.

The reduction of the number of blood vessels was considered as positive indication of anti-angiogenic potential(14). Quantitative analysis of the present study revealed that all embryos treated with the different concentrations of ethanolic extract and ethyl acetate fraction of A. auriculiformis significantly inhibited the outgrowth of new blood vessels in the chorioallantoic membrane. The anti-angiogenic property shown might be due to high phenol content present in extract and fraction. Several polyphenols have reported for their antiangiogenic effects(20).

Many studies suggest that angiogenesis is vital for the development of solid tumors. Angiogenesis is also involved in metastasis by allowing cancer cells to spread from primary tumor site. The development of tumor and metastasis requires angiogenesis because the blood vessels formed provide nutrient and oxygen for the tumor(21). To conclude, inhibition of angiogenesis can control tumor growth and prevent metastasis(14). Antiangiogenesis therapy is a promising approach to the treatment for cancer(21).

Reactive oxygen species (ROS) causes many human diseases including cancer. ROS induces oxidative stress which is involved in the cancer progression. ROS involves in VEGF signaling pathway. VEGF is a very potent angiogenic growth factor that functions in both in both the physiological and pathological conditions. VEGF signaling is essential in normal vascular development and homeostasis. VEGF signaling also promotes the growth of tumor by promoting development of tumor vasculature. Exogenous ROS induces expression of VEGF in many cell types such as endothelial cells, smooth muscle cells, and macrophages. VEGF can raise the level of intracellular ROS. Then, the intracellular ROS induces endothelial cell migration and proliferation. The close relationship between ROS and angiogenesis is proven by extensive studies(22).

The main role of antioxidants is to neutralize free radical to stop the angiogenic effect of ROS(23).Phenolic compounds can neutralize free radicals to reduce the pathological angiogenesis process. Several studies have proven that organic extracts from plant with high content of phenolic compounds are having potent antioxidant

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activities(24).Phenolic compounds can reduce the generation of free radicals by reducing oxidative processes such as lipid peroxidation(21). The capability of inhibiting angiogenesis shown by this extract could be attributed to phenolic compound which have potent antioxidant capacity(21).

Present study proved that the ethanolic extract and ethyl acetate fraction of A. auriculiformis bark have anti-angiogenic effect on CAM model which could be due to its high phenolic content.This observed vascular inhibitory effect of A. auriculiformis suggests its anti-cancer property(14).

4. Conclusion

The ethyl acetate fraction as the phenolic rich fraction was having the highest phenolic content which was equivalent to 621±16.20mg of gallic acid per gram of dried fraction powder. Ethanolic extract of A. auriculiformis and its ethyl acetate fraction showed inhibition in the blood vessel formation in CAM. From the study we concluded that A. auriculiformis plant is a potential source for anti-angiogenic compound. Future studies are planned in the bioassay guided isolation, purification and identification of the anti-angiogenic constituent in the ethyl acetate fraction & ethanolic extract of A. auriculiformis(25). The identification of the active compounds is essential to discover the underlying mechanism of anti-angiogenic activity of A.auriculiformis (24).

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Anti-Angiogenic Effect of Ethanolic Extract and its Phenolic Rich Fraction of *Filicium decipiens* in the Chick Embryo Chorioallantoic Membrane Model

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Abstract

Cancer has been reported to be the 4th most common causes of mortality in Malaysia, in year 2018. Although there are various cancer treatments available, side effects are always be the limitation of these treatments. Various medicinal plants have been studied extensively for their anti-angiogenic activity. Moreover, natural sources are safer and produce lesser side effects. This study aimed to search for alternative cancer treatment from medicinal plant, by examining anti-angiogenic activity of ethanolic extract and its phenolic rich fraction of Filicium decipiens (FD)in the chick embryo chorioallantoic membrane (CAM) assay.

The plant extract was prepared by maceration in 70% ethanol andits fractions (hexane, ethyl acetate and aqueous) were prepared from dry ethanolic extract. Total phenolic content (TPC) of the fraction was assayed by using Folin-Ciocalteu method. CAM in-ovo method was used to evaluate the anti-angiogenic activity of ethanolic extract of FD bark (250µg, 500µg) and its phenolic rich fraction (50µg, 100µg). Prednisone (250µg) was used as positive control. Qualitative observation of reduction in the thickness of blood vessels and quantitative analysis in the reduction of the number of total blood vessels and percentage of blood vessels inhibition were measured to determine the anti-angiogenic activity of the extract and fraction.

Ethyl acetate fraction contained the highest total phenolic content (349.59mg ± 0.29) than aqueous (123.17mg± 0.25), hexane (175.31mg ± 0.18) fractions. Ethanolic extract (250µg, 500µg) and ethyl acetate fraction (50µg, 100µg) showed significant reduction ($P<0.05$) in the total number of blood vessels (43, 14, 46 and 33) compared with negative control (62). Ethanolic extract (250µg and 500µg) and ethyl acetate fraction (50µg and 100µg) showed percentage of blood vessels inhibition of 30.6%, 76.4%, 25.8% and 46.5% respectively. Reduction in the thickness of blood vessels were observed in ethanolic extract (250µg and 500µg) and ethyl acetate fraction (50µg and 100µg).

Ethanolic extract and ethyl acetate fraction of FD bark showed anti-angiogenic activity that may have chemotherapeutic potentials.

Key words : Anti-angiogenic; Filicium decipiens; chick embryo chorioallantoic membrane

1. Introduction

Cancer has been turned up to be the 4th most common causes of mortality in Malaysia in year 2018. There was 12.6% of cancer death reported in government hospital, and this rate was even higher in private hospitals, which was evidenced by a contribution of 26.7% to the death. Overall, cancer had caused 39.3% of death in Malaysia, which is almost half of the death causes. To add on to the previous point, approximately of 37,000 cancer cases are newly diagnosed every year, and it amount will be more than 55,000, by year 2030(1).

Angiogenesis is defined as the generation of new blood vessels from its pre-existing blood capillaries via "sprouting" of endothelial cells which expands the vascular tree. It isregulated and coordinated by variousendogenous systemic or local chemical signals, that are vital in regulating smooth muscle cells as well as endothelial cells function for repairing damaged blood vessels. It is a normal, yet crucial process needed by human body as it helps in human development, reproduction, and wound repair(2).However, angiogenesis is also one of the contributing factors to the development of cancer.

Nowadays, interest has been developed to study and investigate the mode of action of various phytochemicals from different medicinal plants. Several studies have been conducted to reveal their antioxidant activity. Plants such as Tragopogon porrifolius, Lasiosiphonerio-cephalusdecne, Leea indica and other medicinal plants have been studied extensively for their antioxidant

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activity(3,4,5). Natural products or secondary metabolites such as lignans, terpenoids, coumarins, tannins, phenolic acids, quinones, alkaloids, and flavonoids discovered from medicinal plants are showing significant antioxidant effect, which is playing very vital role to the treatment of cancer (6). Phytochemicals such as polyphenolic acids, phenolic diterpenes, tannins, and flavonoids with versatile biological activities are the potential source of natural antioxidants. Plant polyphenols are being recognized as potential choice of therapeutic agents in targeting cardiovascular disease, pathological angiogenesis, and cancer, in the next decade (7). There is strong evidence and recommendations from associated meta-analyses as well as the support from epidemiological studies that people are being protected from diseases such as diabetes, neurodegeneration, osteoporosis, cardiovascular diseases, and cancer development if they consume plant polyphenols-rich diet in a long term basis (8, 9).

Filicium decipiens (FD) originates from the family of Sapindaceae, and its common name in English is known as fern-leaf tree, while in Malaysia, it is known by its local name, Payung. FD grows in tropic zones such as Asia and Africa, whereit can be found in areas with a height up to 1000 meters. It is a 25-meter tall treewith grey brown stem (10). FD had been studied for its antidiabetic, hypolipidemic, anti-inflammatory and antioxidant activity. Its phytochemistry found such as sitosterol, kaempferol and quercetin raised the interest for this study (11). Hence, this study aims to evaluate the anti-angiogenic effect of ethanolic extract and its phenolic rich fraction of Filicium decipiens, which may be a beneficial finding for cancer treatment in the future.

2. Materials and Methods

Chemicals and reagents

70% Ethanol, ethyl acetate, n-hexane, Folin-Ciocalteu reagent, phosphate buffer saline, sodium carbonate, prednisone, and gallic acid were purchased from Medigene Sdn. Bhd. (Selangor)

Collection and authentication of plant materials

The barks of FD were collected in Cheras, Malaysia and authentication of the plant was obtained from Mohd Hafizi Adzmi Hanafi, Biodiversity Unit of University Putra Malaysia (UPM). (UPM/IBS/UB/H24/19)

Preparation of ethanolic extract

Fresh barks of FD were cleaned thoroughly by using distilled water, then dried under shade in a dust-free, clean environment and grinded into fine powder after they were completely dried. Maceration technique was employed by immersing 180g of dried FD bark powder in 70% ethanol, at a ratio of 1:10 for 4 days (12). After 4 days, the ethanolic extract was filtered through a Whatman filter paper, and the filtrate was collected and evaporated by using rotary evaporator (Buchi, R-200 Switzerland). Finally, the resulting concentrated ethanolic extract was lyophilised using freeze dryer, and the dried ethanolic extract was stored in airtight container for further use.

Preparation of phenolic rich fractions

5 g of dried ethanolic extract was weighed and dissolved in 100 ml of water, and then fractionated sequentially using a separatory funnel with 100 ml of hexane and 100 ml ethyl acetate respectively. The resultant fractions were evaporated and concentrated. The different concentration of ethanolic extract and its phenolic rich fractions were used for anti-angiogenic study (13).

Total phenolic content (TPC) assay

Folin-Ciocalteu method was employed to determine and analyse total phenolic content (TPC) of the fractions obtained. 1 ml of sample solution was mixed with 5 mL of Folin-Ciocalteu reagent (diluted tenfold) and incubated in the dark at room temperature for 3?min. Then, 4 mL of saturated 7.5% Na_2CO_3 was added to the mixture and the final volume was 10mL. The mixture was then further incubated for 90 minutes in the dark and its absorbance value was taken at a wavelength of 765?nm using double beam UV spectrophotometer. A standard curve was obtained by mixing 1 mL aliquot of 10 µg/mL, 20 µg/mL, 30 µg/mL, 40 µg/mL and 50 µg/mL gallic acid solution with 5 mL of FolinCiocalteu reagent and 4 mL of $NaCO_3$ solution. The results were expressed as gallic acid equivalents (GAE), which is the amount of gallic acid (mg) per gram of extract. The total phenolic contents of the fractions were calculated by using the formula,(14)

$$C = C1 \times V/m$$

where C = total phenolic content in GAE (mg/g)
C1 = concentration of gallic acid obtained from the calibration curve in (mg/ml)
V = volume of extract in (ml)
m = weight of the plant extract in (g).

The TPC determination of each fraction was performed in triplicate, to get their average result, and the data were reported as mean ± SD.

Preparation of test samples

The stock solution of standard prednisone (10µmg/mL), ethanolic extract (4 mg/0.2 mL), and ethyl acetate

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fraction (1 mg/0.2 mL) were prepared by dissolving in 1% w/v phosphate buffer solution. Filter papers of 5mm diameter were punched out and sterilized properly. Different volume of solutions were applied drop wise on the sterilized filter papers according to the required dose to be tested(15). The concentration of prednisone, ethanolic extract and ethyl acetate fraction used as follow:

Prednisone : 250 µg/pellet (25 µL)

Ethanolic extract : 250 µg/pellet (12.5 µL) and 500 µg/pellet (25 µL)

Ethyl acetate fraction : 50 µg/pellet (10 µL) and 100 µg/pellet (20 µL).

In Vivo chick embryo chorioallantoic membrane (CAM) assay

30 fertilized chicken eggs were purchased from local hatchery, Hing Hong Sdn. Bhd.

Inclusion criteria

- Three days old fertilized fresh eggs.
- Eggs without any sign of cracking.

Exclusion criteria

- More than three days old fertilized chicken embryos.
- Cracked eggs.
- Double yolk embryos.

Pharmacological test

All eggs were cleaned by using 70% ethanol to remove dust and impurities. Then, the eggs were incubated in a horizontal position under a constant humidity around 60% at 37 °C(16). The eggs were divided into the following groups, and each group consists of 5 eggs:

Group 1: Negative control (without drug)

Group 2: Prednisone (250 µg/pellet)

Group 3: Ethanolic extract of FD (250 µg/pellet)

Group 4: Ethanolic extract of FD (500 µg/pellet)

Group 5: Ethyl acetate fraction of ethanolic extract of FD (50 µg/pellet)

Group 6: Ethyl acetate fraction of ethanolic extract of FD (100 µg/pellet)

On incubation day 3, a small hole was created by using a sterile pin at one end of the eggs to remove about 2 to 3ml of albumin by using sterile syringe with needle to detach the developing CAM(16). On incubation of day 4, a square window of about 2cm x 2cm was created by using rotary cutter to expose the blood vessels on the CAMs. Parafilm tape was used to seal the window created and the eggs were continued to be incubated until day 10 of the experiment. All procedures were done under laminar air flow hood.

At day 10 of incubation, different concentration of ethanolic extracts (250 µg/pellet and 500 µg/pellet), its ethyl acetate fractions (50 µg/pellet and 100 µg/pellet) and prednisone (250 µg/pellet) were loaded on sterile filter paper with a diameter of 5mm and placed on the CAM. An egg without applying any test sample was set as negative control of this study.

All procedures were performed under laminar air flow hood to ensure surrounding environment is sterile, to minimize the contamination. CAMs were observed 24 hours later and photographed with a digital camera for detailed images. The thickness of the blood vessels was observed, and the primary, secondary and tertiary blood vessels number were counted and recorded. The result of experiment groups was compared with negative control group. To ensure consistency, the largest blood vessel from the heart was designated as primary blood vessel (PBV), blood vessels that branch out from the primary blood vessel were designated as secondary blood vessels (SBV), and for blood vessels that branch out from the secondary blood vessels are counted as tertiary blood vessels (TBV)(17). The total blood vessels (TTV) were calculated by adding PBV, SBV and TBV together. The following formula was employed to calculate the percentage of inhibition(18).

Percentage inhibition= [(N of CAM treated in negative control group - N of CAM treated by extracts/ fraction) / (N of CAM treated in negative control group)] × 100%

Whereby N= Total blood vessels (TTV)

Statistical analysis

The data was analysed using one-way ANOVA followed by Tukey's post hoc test, and all results were recorded as mean ± standard deviation. The results were considered significant when P value is <0.05.

3. Results and Discussions

Total phenolic content

TPC of each fraction was calculated by using the standard curve with the equation of y = 0.0140x + 0.0459, where R2 = 0.9993 (Graph 1), and the values were expressed as mean ± standard deviation as shown in Table 1.

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It was shown that ethyl acetate fraction (0.1 mg/mL) contains the highest phenolic content compared to water (0.1 mg/mL) and hexane fractions (0.1 mg/mL). (Table 1) Ethyl acetate fraction contains about twice the amount of phenolic contents (349.59 ± 0.29mg) than hexane fraction (175.31 ± 0.18mg), and almost thrice the amount of phenolic content in water fraction 123.17 ± 0.25mg). Water fraction contains the lowest amount of phenolic content among the three fractions(19). (Table 1)

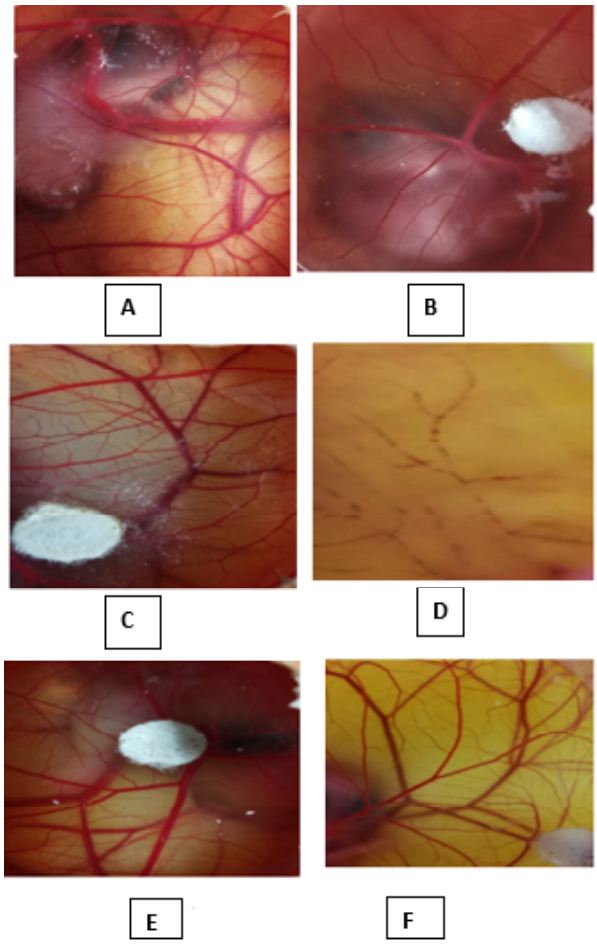
Table 1. TPC of water, hexane and ethyl acetate fractions

Fractions	TPC (GAE, mg/g)
Water	123.17± 0.25
Hexane	175.31 ± 0.18
Ethyl acetate	349.59 ± 0.29

Chick Chorioallantoic Membrane Assay

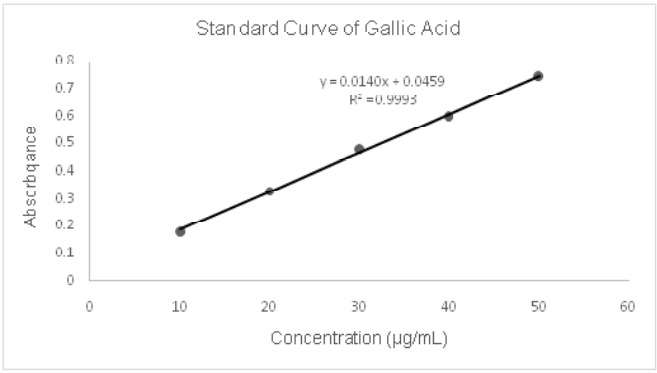
Qualification and Quantification of Blood Vessels

The condition and thickness of blood vessels in CAM treated by different groups were observed and shown. (Figure 1 A-F) There was significant reduction (P<0.05) in the thickness of the blood vessels in prednisone (250µg), ethanolic extract (250 µg, 500 µg) and ethyl acetate fraction (50 µg, 100 µg) treated group, when compared to the negative control group. Different concentrations of extracts and fractions showed different extent of reduction in the thickness of blood vessels. (Figure 1 A-F) The number of primary, secondary, tertiary blood vessels and percentage of inhibition were calculated.



CAM treated with negative control (A), 250µg of prednisone as positive control (B), 250µg of ethanolic extract (C), 500µg of ethanolic extract (D), 50µg of ethyl acetate fraction (E) &100µg of ethyl acetate fraction (F).

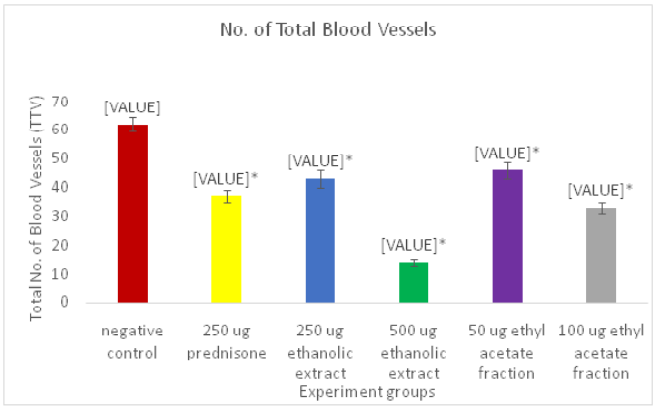
Fig 1: Image of CAM from each treatment groups.



Graph 1. Standard curve of gallic acid

The presence of bioactive compounds, which is known as secondary metabolites is always associated with medicinal and pharmacological actions of medicinal plants. Secondary metabolites like alkaloids, waxes, terpenoids, fatty acids, phenolics (simple phenolics and flavonoids), glycosides and their derivatives are reported to have medicinal properties, such as inhibition of the process of angiogenesis(20). Angiogenesis contributes to the growth and metastasis of tumor as it supplies essential nutrients oxygen needed by the growing tumors.

As supporting evidence to the potential anti-angiogenic activity of FD, other studies have



Graph 2. Number of blood vessels among different experiment groups

* means significant results with p<0.05compared with negative control group.

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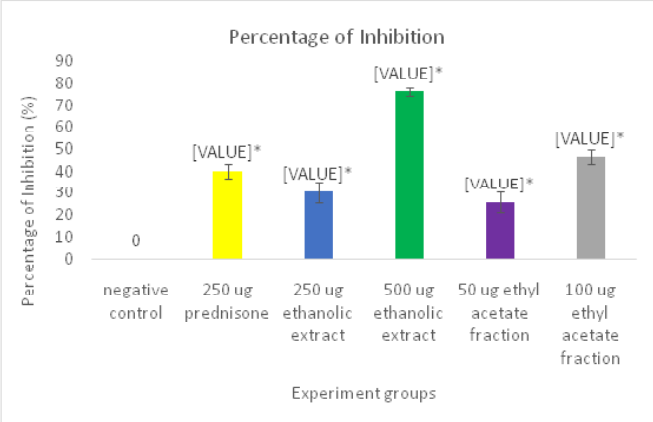

Graph 3. Percentage of blood vessels inhibition among different experiment groups
* means significant results with p<0.05compared with negative control group.

demonstrated that selected phytochemical constituents (phenolics) extracted from this plant showed anti-oxidant activity (21). Furthermore, a correlation between antioxidant and anti-angiogenesis was observed, and it is reported that angiogenesis in vitro is stimulated by vital endogenous reactive oxygen species (ROS), for example, hydrogen peroxide. Too much ROS will give rise to oxidative stress, and hence promoting various human ailments such as inflammation, hypertension, atherosclerosis, as well as cancer(17). Antioxidants can scavenge reactive oxygen species which damage lipids, proteins and DNA.

In CAM, chicken allantoic fluid secretion provides natural endogenous growth factors to it. These proteins play important roles in blood vessels growth during development of chicken embryo(22). In my study, ethanolic extract (250 µg, 500 µg) and its ethyl acetate fraction (50 µg, 100 µg) of FD showed significant reduction ($p<0.05$) in the thickness as well as the number of total blood vessels and percentage inhibition in the chick embryo chorioallantoic membrane (CAM) assay (Graph 2& 3). Its anti-angiogenic activity may due active principles that are present in ethyl acetate fraction, which are mostly semi-polar phenolic compounds(23), while antiangiogenic effect of ethanolic extract showed that, apart from the phenolic compounds, there are still other phytoconstituents work synergistically with phenolic compounds to exhibit a significant reduction in blood vessels vascularization(24). According to Usman et al., phenolic compounds are able to inhibit angiogenesis process by inhibiting VEGF expression, migration of endothelial cell, as well as decreasing matrix metalloproteinases(17).

Based on the present study, by using chick embryo CAM model, the new pharmacological effect of FD has been confirmed, proven by the inhibition of angiogenesis in term of total blood vessels number, as well as thickness of the blood vessels. FD bark ethanolic extract and its ethyl acetate fraction showed significant antiangiogenic activity at all doses of treatment studied, where both ethanolic extract and ethyl acetate fraction reduced the thickness and number of total blood vessels in CAM. To date, there have been extensive studies on natural product compounds as well as extracts that showed potent anti-angiogenic activity, in conjunction to having good antioxidant activities(17). We can therefore conclude that the anti-angiogenic activity of ethyl acetate fraction from FD bark in this study may arise from its anti-oxidative capacity, primarily due to the presence of phenolic compounds(17, 23), whereby antiangiogenic effect of ethanolic extract from FD may due to the presence of other phytoconstituents in addition to phenolic compounds(24).

4. Conclusion

The significant reduction in the thickness and total number of blood vessels revealed the anti-angiogenic activity of ethanolic extract and its ethyl acetate fraction of FD. The anti-angiogenic activity of ethanolic extract and its ethyl acetate fraction are due to the presence of phenolic compounds. Hence, it can be concluded that FD could provide a new source of chemical agents for anti-angiogenic cancer therapy and warrants further studies.

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Knowledge and Awareness About Blood Pressure, Stroke and Prevalence of Hypertension : A Cross-Sectional Study in a Private University, Kuala Lumpur

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Abstract

Prevalence of hypertension is rising in Malaysia. This study aimed to assess the prevalence of hypertension among students and staff of a private university in Malaysia. As most of the risk factors of hypertension is modifiable in nature, this study also aimed to determine the knowledge of university students and staff on hypertension, stroke, and linkage between these two diseases. Convenient sampling used to conduct a survey during a blood pressure screening campaign. A total of 803 responds were collected, and the prevalence of hypertension was found to be 5.5% (45 out of 803 respondents). Hypertension was more prevalent in male respondents (9.1%) than female respondents (1.8%) ($P < 0.05$). Other factors associated to hypertension include smoking habits and high body mass index (BMI). Weak positive correlations were observed between the BMI category and blood pressure (r=.396 for systolic blood pressure, r=.317 for diastolic blood pressure). 17.3% of male respondents were found to be active smokers, while 34.9% of total respondents were either overweight or obese. The overall knowledge of students and staff on hypertension and stroke was good, however, certain knowledge insufficiencies were identified. Factors affecting knowledge includes the educational field of students and the academic qualifications of respondents. As a conclusion, our study found knowledge gap and potential interventions which university and healthcare team may target to reduce the prevalence of high blood pressure in university settings.

Keywords : hypertension knowledge, stroke knowledge, hypertension prevalence, university

1. Introduction

Hypertension is one of the most common medical conditions seen in primary healthcare settings. It may lead to myocardial infarction, stroke, renal failure, and premature death if not detected and treated early.(1) Lewington S *et al*. reported that an increase in 20mmHg systolic blood pressure and an increase in 10mmHg of diastolic blood pressure may double the risk of mortality caused by stroke and ischemic illnesses.(2) As a developing country, Malaysia's population is still trying to adapt the unprecedented economic growth and modernization in which both directly and indirectly affect the health-related behaviour of the population.(3)

The prevalence of hypertension in Malaysia has been increasing for the previous years.(3) A Malaysian study in 2016 reported that up to 32.7% of Malaysian adults have hypertension.(4) Due to the significant health care burden of hypertension, its increasing prevalence indicates that more attention in Malaysian healthcare community must be allocated to this non-communicable disease.

Various factors, such as high salt intake, excessive adrenergic tone, sedentary lifestyle, genetic influences, poor sleep habits, smoking, alcoholism, and others have been identified to be associated with essential hypertension. (5-7) Furthermore, other demographic differences such as educational level, gender, and body mass index (BMI) are also associated with increased blood pressure. (8,9) As most of the risk factors are modifiable in nature, it is vital for researchers to assay the population's awareness and knowledge in identifying new strategies to reduce prevalence of hypertension.

Gooding HC and colleagues reported that most of the young adults had poor awareness of hypertension, and this puts them at increased risk of this disease.(10) Another investigation by Zhang YY and Moran AE found that adults aged 18 to 39 had significantly lower awareness of hypertension when compared to elder adults.(11) They also documented that male participants had generally lower hypertension awareness than female participants. (11) From our literature reviews, there are insufficient

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studies or reports on the prevalence of hypertension in adults from a university setting in Malaysia.

Hence, this study aimed to investigate the prevalence of hypertension among university students and staff. The study also assessed the current level of knowledge and awareness about hypertension and stroke among the study population, as well as the relationship of hypertension to stroke.

2. Materials and Methods

Study design

This was adescriptive cross-sectional study conducted at UCSI University, Malaysia. In this study, a blood pressure screening campaign was conducted from June to July 2018 to measure the blood pressure of university students and staff. During the campaign, all participants aged 18 years old and above were presented with Respondent Information Sheet and invited to fill up the questionnaire. Participants who refused to provide informed consent were excluded from the study.

The screening processes were carried out by undergraduate pharmacy students under the supervision of pharmacists. The students were trained to follow the blood pressure measurement protocol specified by the Malaysia Society of Hypertension (MSH) before the commencement of screening campaign. The blood pressure was measured from participants' non-dominant arm twice at 1 minute apart, and the mean values were taken as participants' blood pressure. If the difference between the first and second readings are 20 mmHg or greater, then a third reading was taken. To ensure consistency of the measurements, 'Rossmax X5 Digital Blood Pressure Monitor' was used throughout the research.

Sample size and sampling technique

During the sampling period, there were around 2500 active science stream and 3000 art stream students in UCSI University, with 362 academic staff and 326 non-academic staff. By using a margin error of 0.05 at 95% confidence interval, the computed sample size for science and art stream students were 334 and 341 subjects respectively. The sample size required for academic and non-academic staff were 187 and 177 subjects respectively. Convenient sampling was used to attain the participants.

Research instrument

The questionnaire adopted from MSH is available in English, Malay, and Chinese. It consisted of 53 questions in four sections. Section one comprised of 11 questions on participants' demographic data, including field of study, gender, smoking status, and others. Section two assessed participants' most recent blood pressure measurement and current health status. Section three had 16 questions evaluating participants' knowledge of hypertension, while section four evaluated participants' knowledge of stroke. For the last two sections, one mark was given for each correct answer, and no mark was given for incorrect answers.

The questionnaire's face and content validity were assessed by expert reviewers, and a pilot test was conducted on 50 samples to determine its reliability. The final iteration of questionnaire was disseminated to the eligible respondents who gave their informed consent for this study.

Respondents who replied more than 75% correct answers for the knowledge questions were deemed to have good knowledge in that section. Respondents who scored 50% to 74% correct answers were categorized as having moderate knowledge, while those who scored less than 50% were categorized as having poor knowledge.

Data analysis

Data was entered in Microsoft Excel 2016 and analysed using Statistical Package for the Social Science (SPSS) software version 23. Descriptive data such as demographic characteristics were described as percentages. The sample population's average blood pressure and knowledge scores were described as mean with 95% confidence interval (CI).

Mann-Whitney U Test, Kruskal Wallis Test, and Chi-Square Test were used to compare between groups, and Spearman's correlation was used to measure association between factors. A P-value of $P > 0.05$ is considered to be statistically significant.

3. Results and Discussion

Demographic data, blood pressure, and BMI of respondents

A total of 677 students and 126 staff consented to the study. 51.8% of respondents were male (n = 416). Majority of respondents are non-smoker (88.6%), while 59.2% did not drink any alcoholic beverage.

Blood pressure profile of respondents were classified according to the Malaysia Clinical Practice Guideline: Management of Hypertension 5th edition.(12) From Table 1, 45 out of 803 respondents (5.5%) had systolic blood pressure exceeding 140mmHg and/or diastolic pressure

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exceeding 90mmHg. The average systolic blood pressure of the study population was 117.24mmHg [95% CI: (116.24,118.23)] and the average diastolic blood pressure was 74.12mmHg [95% CI: (73.47,74.78)].

Table 1: Demographic data, blood pressure, and BMI of respondents

Variable	
University student (n, %)	677 (84.3)
• Science stream	334 (41.6)
• Art stream	343 (42.7)
University staff (n, %)	126 (15.7)
• Academic staff	67 (8.3)
• Non-academic staff	59 (7.3)
Mean age in years	
• Students	21.8
• Staff	36.6
Gender (n, %)	
• Male	416 (51.8)
• Female	387 (48.2)
Alcoholic beverage drinker (n, %)	
• Non-drinker	328 (59.2)
• Social drinker	289 (36.0)
• Moderate drinker	34 (4.2)
• Heavy drinker	5 (0.6)
Smoking status (n, %)	
• Non-smoker	712 (88.6)
• Social smoker	64 (8.0)
• Moderate smoker	17 (2.1)
• Heavy smoker	10 (1.2)
Blood pressure classification (n, %)	
• Optimal	470 (58.5)
• Normal	190 (23.7)
• At risk	98 (12.2)
• Stage 1	38 (4.7)
• Stage 2	6 (0.7)
• Stage 3	1 (0.1)
Body mass index (n, %)	
• Underweight	128 (15.9)
• Normal	392 (48.8)
• Overweight	200 (24.9)
• Obese	78 (9.7)

Table 2 shows respondents' blood pressure category based on their gender and occupation. The average systolic/diastolic blood pressure of university staff was 120.61/79.19 mmHg [95% CI: Systolic (117.86,123.36), Diastolic (77.39,80.99)], which is higher than that of students' 116.61/73.18 mmHg [95% CI: Systolic (115.55,117.67), Diastolic (72.51,73.86)] ($P < 0.05$). A possible reason for such observation could be due to the overwhelming workload and emotional stress of university staff. A study by Adedoyin RA et al. observed that university staff were often occupied by lecture classes with tight schedule, meetings and other duties, leading to psychological stresses which contribute to an increase in blood pressure.(13)

Table 2 : Blood pressure of respondents based on gender and occupation.

Variable	Gender		Population	
Blood Pressure Classification (n, %)	Male (n = 416)	Female (n = 387)	Students (n = 677)	Staff (n = 126)
• Optimal	174 (41.8)	296 (76.5)	407 (60.1)	63 (50.0)
• Normal	130 (31.3)	60 (15.5)	167 (24.7)	23 (18.3)
• At risk	74 (17.8)	24 (6.2)	69 (10.2)	29 (23.0)
• Stage 1	31 (7.5)	7 (1.8)	28 (4.1)	10 (7.9)
• Stage 2	6 (1.4)	0 (0)	5 (0.7)	1 (0.8)
• Stage 3	1 (0.2)	0 (0)	1 (0.1)	0 (0)

For university students, the prevalence of hypertension was 4.9% (n = 34). This finding was lower than a previous study from Shah Alam, Malaysia, where 10% of the students were found to be hypertensive.(14) Nevertheless, it is alarming to see that 1 in 20 young adults in this university were already having high blood pressure.

Our data also found that high blood pressure was more prevalent in male (9.1%) than female respondents (1.8%) ($P < 0.05$). The mean systolic and diastolic blood pressure of male respondents was 122.68mmHg [95% CI: (121.35,124.02)] and 75.44mmHg [95% CI: (74.51,76.37)] respectively, whereas systolic and diastolic blood pressure for female was 111.39mmHg [95% CI: (110.14,112.63)] and 72.71mmHg [95% Cl: (71.81,73.60)] ($P < 0.05$).This finding is consistent with other prevalence studies in Malaysia which reported higher hypertension prevalence among men than women.(14-16) A previous study reported that differences in blood pressure levels can be evident among males and females even in their twenties.(17) Possible factors include the better BMI profile of female, and the lesser likelihood of female becoming a smoker.(17) Indeed, in our study there were more smokers among male respondents than female (17.3% of male vs 4.9% of female respondents) ($P < 0.05$). The difference in body composition and insulin resistance has also been

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suggested as contributing factors for lower blood pressure among female adolescents.(8) Furthermore, estrogen may act as a protective factor against hypertension.(18)

Smokers were found to have higher systolic blood pressure than non-smoker ($P < 0.05$). Possible reasons include the enhanced atherogenesis in large capacitance vessel among smokers, leading to increased systolic blood pressure.(19) Nicotine may also increase peripheral vasoconstriction via sympathetic pathway, and increase serum lipid level which gives rise to atherosclerosis.(20) The percentage of smokers in our study population (17.3% of male respondents) suggested that smoking cessation campaign can be beneficial in university settings. The negative consequences of smoking had been well-documented in most of the practice guidelines around the world, but smokers who volunteered for smoking cessation remained low in Malaysia.(21) University can play a role by establishing policies or enforcing rules that include punitive measures on students who were found to be smoking in the campus. Education and reminders should be given to students on frequent basis. Furthermore, abolishing smoking area in university for students to smoke can be effective as well.

Our study did not find any significant difference in blood pressure across different categories of alcohol consumption. This finding is dissimilar with other studies which reported dose-related relationship between alcohol consumption and blood pressure.(22) It is possible among the drinkers in our study population, majority (88%) of them were light drinker, possibly due to financial restriction of university students in obtaining costly alcoholic drinks.

BMI profile of respondents were classified according to Malaysia Clinical Practice Guidelines: Obesity.(23) The mean BMI of university students was 21.70kg/m2 [95% CI: (21.40,21.99)] and the mean BMI of university staff was 24.88kg/m2 [95% CI: (24.10,25.65)] ($P < 0.05$). Male respondents have significantly higher BMI value than female respondents ($P<0.05$). The average BMI for male and female was 22.85kg/m2 [95% CI: (22.45,23.27)] and 21.49kg/m2 [95% CI: (21.11,21.87)] respectively. This could be one of the contributing factors for higher blood pressure level among male respondents.

Correlation between blood pressure and BMI

Table 3 shows that as BMI class increased, the average systolic and diastolic BP increased. The blood pressure level was significantly different in each class of body mass index ($P < 0.05$).

Table 3 : Average systolic and diastolic blood pressure in each BMI category.

Variable	Average systolic BP (mm Hg)	Average diastolic BP (mm Hg)
Body mass index		
• Underweight	110.12 (108.04,112.20)*	70.92 (69.52,72.32)*
• Normal	115.06 (113.80,116.33)*	72.48 (71.68,73.29)*
• Overweight	121.22 (119.28,123.15)*	76.41 (75.04,77.77)*
• Obese	129.46 (125.79,133.14)*	82.08 (79.58,84.58)*

*Values presented as mean with 95% confidence interval.

Variable Average systolic BP (mm Hg) Average diastolic BP (mm Hg)

Spearman's correlation was carried out to determine the relationship between average blood pressure level with BMI. Result revealed statistically significant positive correlation between average systolic and diastolic blood pressure with body mass index. ($P < 0.05$) Weak positive correlation was found between BMI and average systolic blood pressure ($r=.396$), and between BMI and average diastolic blood pressure ($r=.317$).

This is similar to the findings from REDISCOVER Study of hypertension, where obese respondents were reported with higher prevalence of high blood pressure.(4) BMI has been reported to be a good predictor of blood pressure, where an increment of 1 unit BMI can contribute to an average increase of 2.0mmHg in systolic blood pressure and 1.3mmHg in diastolic blood pressure.(24)

High BMI is one of the common modifiable risk factors for many disease, and appropriate prevention can help to reduce healthcare burden to the society. Besides, physical inactivity, poor dietary habit may also contribute to the high BMI levels.

National Health and Morbidity Survey 2015 reported that Malaysians had poor daily intake of vegetables and fruits.(21) Students' hectic and heavy workload may be associated with unhealthy eating practice too. Deliens T. and colleagues documented that university students' busy involvement in faculty events and academic activities may have driven students to neglect healthy diet, and seek for fast and convenient food.(25) Although Malaysian Dietary Guideline is available for the public, many Malaysian were not aware of it.(26) Healthcare professionals and lecturers should take a more active role in promoting healthy diet to students and public.

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Knowledge about hypertension

The knowledge about hypertension was also compared between science stream and art stream students. From Table 4, 81.1% of science stream students have good knowledge, compared to only 72.3% of art stream students. Mean knowledge score for art stream and science stream students was 12.25 [95% CI: (11.94,12.56)] and 13.29 [95% CI: (13.03,13.55)] respectively ($P < 0.05$). This may be due to the exposure of science stream students to biology subjects and human physiologies in the academic curriculum.

Table 4 : Respondents' knowledge on hypertension

Variable	Student		Staff	
Knowledge on hypertension (n, %)	Art Stream (n = 343)	Science stream (n = 334)	Academic (n = 67)	Non-academic (n = 59)
• Poor	25 (7.2)	12 (3.6)	2 (3.0)	0 (0)
• Moderate	70 (20.4)	51 (15.3)	4 (6.0)	17 (28.8)
• Good	248 (72.3)	271 (81.1)	61 (91.0)	42 (71.2)

Additionally, there are more academic staff (91.0%) who had good knowledge when compared to non-academic staff (71.2%) ($P < 0.05$).This may be explained by the differences in education level between academic and non-academic staff. 73.2% of the academic staff have post-graduate qualifications, while 71.2% of non-academic staff only attained academic qualifications of bachelor's degrees or below.

Smokers and alcoholic drinkers were not found to have significant differences in knowledge when compared to their respective counterparts.

The study also investigates respondents' responses to individual questions. For the question relating high salt intake to risk of hypertension, the majority of the study population (n = 637, 79.3%) answered correctly. However, among university staff, it was discovered that 30.5% of the non-academic staff were not aware that high salt intake is one of the risk factors of hypertension. Furthermore, it was found that among 166 respondents who were not aware that high salt intake can increase the risk of hypertension, 42.2% of them were either overweight or obese. This suggested poor dietary awareness.

86.7% of study population were aware that frequent stressful lifestyle can increase the risk of hypertension. There was significant difference between science stream and art stream students ($P<0.05$) on the choice of answer for this question, where 88% of science stream answered correctly but only 80.8% of the art stream students chose the right answer ($P < 0.05$). There was also significant difference between university staff and students ($P < 0.05$), where 15.6% of university students and 7.1% of university staff considered stressful lifestyle to not affect blood pressure level. Good stress management practice should be instilled in all university students and staff. University students are susceptible to high level of stress due to multiple examinations, overwhelming homework and other assignments.(27) Nichter M. discovered that students has a tendency to take cigarette smoking in an attempt to handle stress.(28) Such unhealthy practices should be stopped and prevented at the university level through proper education and awareness.

Majority of the respondents (55.2%) were not aware that a sedentary lifestyle can increase the risk of hypertension.Only 39.4% of student respondents answered this question correctly, compared to 65.1% of staff ($P < 0.05$). This shows a poor awareness of the study population on the linkage between sedentary lifestyle and high blood pressure.

771 respondents (96%) were aware that the public should measure their blood pressure at least once per year. However, out of these 771 respondents, only 54.6% of them did measure their blood pressure within past one year. Besides, a small percentage of them (9.9%) never had their blood pressure measured before, even though they believed that it is important to measure blood pressure on yearly basis. Many teenagers and young adults are prone to have poor attitudes and practices for

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Table 5 : Respondents' answer on selected hypertension knowledge questions

Question	Science Student (n = 334)		Art Students (n = 343)		Academic Staff		Non-academic Staff	
(n, %)	Correct	Incorrect	Correct	Incorrect	Correct	Incorrect	Correct	Incorrect
Risk factors of hypertension								
High salt intake	272 (81.4)	62 (18.6)	254 (74.1)	89 (25.9)	60 (89.6)	7 (10.4)	41 (69.5)	18 (30.5)
Stressful lifestyle	294 (88.0)	40 (12.0)	277 (80.8)	66 (19.2)	62 (92,5)	5 (7.5)	55 (93.2)	4 (6.8)
Sedentary lifestyle	152 (45.5)	182 (54.5)	115 (33.5)	222 (66.4)	45 (67.2)	22 (32.8)	37 (62.7)	22 (37.3)
BP Screening								
Is it important to measure BP at least once a year?	317 (94.9)	17 (5.1)	330 (96.3)	13 (3.7)	67 (100)	0 (0)	57 (96.6)	2 (3.4)

health screenings, they believe that most of the non-communicable diseases are age-related rather than lifestyle-related. Evans N. and colleagues revealed that the health risks possessed by adolescence these days were unmatched with their health priorities.(29) In another study conducted by Cao QQ. and colleagues, they reported that many patients only found out that they had hypertension when stroke occurred.(30)

Most of the respondents (87.2%) were able to identify stroke as a potential complication of hypertension. There was no statistically significant difference in blood pressure of responders who were aware of such linkage, and those who were not aware of it. However, we did observe a trend where respondents who were aware of such complications are more likely to have optimal blood pressure (59.4%) compared to respondents who disagreed (53.2%). Understanding the complication of chronic diseases is an important factor in active prevention of the diseases.

Knowledge about stroke

77.4% of university students had good knowledge about stroke (77.4%) with a mean knowledge score of 14.24 [95% CI: (13.99,14.50)] out of a total of 19 questions. Similarly, university staff were able to answer knowledge-based questions about stroke as well. Majority of the university staff (80.2%) have good knowledge about stroke. In comparison of stroke knowledge score, university staff {14.89 [95% CI: (14.37,15.40)]} has greater knowledge score than university students ($P < 0.05$).There was no significant difference between knowledge score of academic and non-academic staff.

Science stream students (mean knowledge score 14.63 [95% CI: (14.30,14.96)]) had significantly greater knowledge than art stream students (mean knowledge score 13.86 [95% CI: (13.47,14.25)]) ($P < 0.05$).This is similar for the knowledge of hypertension in current study.

Our study found that 3.4% of student respondents (n = 23) thought stroke affects kidney, lung, or liver.19.9% of the students and 15.9% of staff thought that stroke primarily affects the heart rather than the brain. This was consistent with studies conducted in India and Nepal whereby the knowledge on the stroke-affected organ was poor.(31,32) The observation in our study may be due to a lack of educational campaigns on stroke awareness in Malaysia.

Table 6 : Respondents' knowledge on stroke

Variable	Student		Staff	
Knowledge on stroke (n, %)	Art Stream (n = 343)	Science stream (n = 334)	Academic (n = 67)	Non-academic (n = 59)
• Poor	22 (6.4)	13 (3.9)	2 (3.0)	1 (1.7)
• Moderate	63 (18.4)	55 (16.5)	8 (11.9)	14 (23.7)
• Good	258 (75.2)	266 (79.6)	57 (85.1)	44 (74.6)

Majority of the respondents (87.7%) agreed that lowering blood pressure can reduce the risk of stroke. This showed good awareness on the link of high blood pressure to stroke. According to RE-LY trial, every 10 mmHg increase in mean BP will lead to 6 to 7% increased risk of stroke.(33) It was hoped that as the public aware on the linkage between blood pressure and stroke, they can provide better self-discipline in management of hypertension. In fact, among all common risk factors of stroke, hypertension can be considered as the easiest risk factors to be managed.(34)

87.9% of respondents also understood that stroke patients should be sent to hospital immediately, ideally within the first 4 and a half hours after stroke occurred. However, for respondents who were not aware that brain is the primary organ affected by stroke, half of them (49.7%) did not feel that it is necessary for stroke patients to be admitted immediately. One of the first line

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management of stroke, alteplase, provides better outcome and health benefits if patients were admitted within the first 4 and a half hours.(35) It is important to increase public awareness on this golden hour of stroke treatment.

This study found that the knowledge about hypertension and stroke of university students and staff were good and there was no difference in the blood pressure level between different level of knowledge about hypertension and stroke. This suggested that knowledge level was not associated with raised blood pressure among university staff and students, therefore future study should also assess the attitude and practice of study population to investigate other reasons of the poor health practices.

4. Conclusion

Overall, 5.5% of university staff and students had systolic blood pressure exceeding 140mmHg and/or diastolic blood pressure exceeding 90mmHg. Male respondents have significantly higher average blood pressure than female respondents. Smoking habit and BMI were also found to factors associated with raised blood pressure in university settings.

The prevalence of overweight and obese in university was high. In order to reduce prevalence of hypertension, the importance of a healthy diet and smoking cessation should be regularly disseminated to university students and staff.

University students and staff were found to have overall good knowledge on stroke and hypertension. However, knowledge insufficiency was found in certain areas, such as the link between sedentary lifestyle and hypertension. Science stream students have significantly higher knowledge than art stream students on these healthcare-related topics. As for university staff, the knowledge about hypertension and stroke was found to be significantly higher in academic staff compared to non-academic staff. This may be due to the higher level of education and knowledge sharing culture in academic staff which provide them extra knowledge.

Overall, our study found knowledge gap and potential interventions which university and healthcare team may target to reduce prevalence of high blood pressure in university settings.

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Investigation on Antibacterial Activity of Pyrogallol in Methicillin Resistant *Staphylococcus aureus*

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Abstract

The aims of this study areto study the antibacterial activity of pyrogallol towards MRSA strains and evaluate the effects of various concentrations of pyrogallol over time in relation to the stages of the growth of the bacteria. Antibiotic susceptibility of pyrogallol was assessed using disc diffusion and microbroth dilution method. It was found that the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of pyrogallol were 15.6 µg/mL. Time-kill kinetic assay performed showed that lower concentration of pyrogallol could exhibit some extent of bacteriostatic effect towards the bacteria, whereas higher concentrations were lethal from lag phase onwards. Pyrogallol exhibited strong antibacterial activity against MRSA. It exhibited MIC and MBC at 15.6 µg/mL. Time-kill kinetic assay showed pyrogallol could slightly inhibit the exponential growth of MRSA, and bacteria was lethal at higher concentrations. Possible bactericidal mechanism of pyrogallol was thoroughly discussed in this study. More detailed studies on the mechanism of action is in progress.

Key words : Pyrogallol; Methicillin resistant Staphylococcus aureus; antibacterial; polyphenols; mechanism

1. Introduction

Antibiotic resistance bacteria infections have become an alarming concern and widely spread around the world. Bacteria developed resistance towards antibiotics. This causes massive increment mortality caused by infectious diseases. Methicillin-resistance Staphylococcus aureus (MRSA) has higher mortality rate than human immunodeficiency virus (HIC) or acquired immune deficiency syndrome (AIDS) (1). There were more than 80000 severe infections reported in the USA in 2011. Also more than one-half of hospital-related S. aureus infections reported in most Asian countries were found to be related to MRSA infection (2). The prevalence of MRSA infection remains high in Asia countries due to the poor awareness on the appropriate usage of antibiotics. Ongoing development of new antibiotics, active surveillance efforts and advances in infection prevention are urgently required as MRSA remains a prominent pathogen with persistently high mortality (2). Massive research efforts on the discovery on effective antibiotics against MRSA is needed to combat successive waves of resistant pathogens and to meet the challenges of resistance development.

Antibiotics from natural products are reported to have complex architectural scaffolds and active functional groups which could interact with biological targets. It is believed that natural product-derived antibiotics are synthesized as secondary metabolites due to survival advantages to the organisms. These metabolites are synthesized as organism defense mechanism against pest and pathogens. Natural derived antibiotics could inhibit microoganisms by four classical targets, namely bacteria cell-wall biosynthesis, protein biosynthesis, DNA replication and folate coenzyme biosynthesis (3-5). Examples of natural products derived antibiotics are polymyxin B, valinomycin, daphtomycin, novobiocin, erythromycin, etc.

Pyrogallol (IUPAC name 3,4,5-trihydroxybenzoic acid) is present naturally in numerous plants (Fig 1). It is

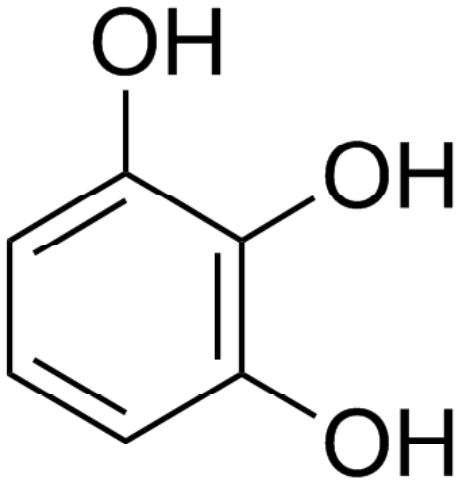

Figure 1 Chemical structure of pyrogallol

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also an important functional group in many polyphenol compounds. Literatures have reported that polyphenols which carry pyrogallol group have stronger bioactivities, compare to those which consist of similar structures, i.e. catechol and resorcinol rings (6-8). It has been reported that pyrogallol moieties in polyphenols is responsible to exhibit broad spectrum antibacterial activity. VN Lima et al (9) recently reported that pyrogallol could synergistically exhibit stronger antimicrobial activity with antibiotics against S. aureus and Candida spp. Although literatures have reported that pyrogallol could exhibit antimicrobial activity towards various bacteria, studies on the antimicrobial action and killing pattern of pyrogallol on MRSA are still not well defined.

The main objectives of this study are to study the antibacterial activity of pyrogallol towards MRSA strains and evaluate the effects of various concentrations of pyrogallol over time in relation to the stages of the growth of the bacteria.

2. Materials and Methods

Bacteria culture

Pyrogallol (Sigma Aldrich) was tested on two strains of methicillin resistance Staphylococcus aureus (MRSA): ATCC 33591 and a hospital strain, gifted by Assoc. Prof. Dr. Vasantha Kumari Neela from Universiti Putra Malaysia. Both bacteria strains were cultivated onto nutrient agar (Oxoid) at 37 °C for 16- 20 hours.

Antibacterial susceptibility assays

Antibacterial susceptibility of pyrogallol was tested with agar diffusion assay (10) and microbroth dilution method (1). In disc diffusion assay, 0.1 mg of pyrogallol, dissolved in 100 µL methanol was loaded onto sterile blank disc (6 mm diameter; Oxoid) and the disc was impregnated onto Mueller Hinton agar (MHA; Oxoid), pre-inoculated with bacteria 1 × 108 coliform units (cfu)/ mL, standardised using Miles and Misra technique (1). The plates were then incubated for 16 - 20 hours at 37°C and the diameter of the inhibition zones was measured. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of pyrogallol was assessed using microbroth dilution method. MIC was recorded as the lowest concentration of pyrogallol which completely inhibit bacteria growth. MBC was determined when no visible growth seen on the first streak on MHA of the clear wells. The test was repeated three times.

Time-kill kinetics assay

Time-kill kinetic assay was performed as described by Kubo et al.(11) and Kang et al (12) with slight modifications. Pyrogallol was diluted with sterile distilled water to achieve 4 different concentrations: ¼ MIC, ½ MIC, MIC and 2 MIC. Pyrogallol at these four concentrations and control group were added into an initial inoculum of 1 × 108cfu/mL. All samples were incubated at 37^0C. Samples were withdrawn at selected time points (0, 4, 6, 8, 10, 12 and 24 hours), serially diluted using sterile saline before samples were plated onto MHA. The plates were then incubated at 37^0C for 20 hours, and colony forming units were estimated. Triplicates were performed for each sample.

Statistical analysis

The experimental results for disc diffusion were expressed as mean ± standard deviation. The data were analysed using one-way analysis of variance (ANOVA) using SPSS version 20.

3. Results and Discussion

Disc diffusion assay demonstrated that both strains of MRSA were susceptible to pyrogallol at 0.1 mg, and the MICs determined were 15.6 µg/mL. MBC for MRSA ATCC 33591 was 15.6 µg/mL. MRSA hospital strain was slightly more resistant to pyrogallol, MBC was 31.3 µg/ mL.

Time kill studies was performed on MRSA ATCC 33591 at four increasing concentrations of pyrogallol to determine the killing pattern and time required. Time kill assays could evaluate the activity of pyrogallol against the MRSA and determine the bactericidal or bacteriostatic activity of an agent over time in relation to the stages of the growth of the bacteria (at lag, exponential, stationary phase).

It was found that pyrogallol slowed down the growth at lower concentrations (¼ MIC and ½ MIC) but could not completely inhibit and kill the microorganism. Longer lag phase was noticed and the number of cells increased after 6 hours of treatment (Fig 2). Although stationary phase was noticed in growth curve for ¼ MIC and ½ MIC, the total number of cells was significantly lower than the control. This showed that lower concentration of pyrogallol could exhibit some extent of bacteriostatic effect towards the bacteria.

Reduction in cells number was observed after treatment at MIC and higher concentration. The pattern of antibacterial activity of pyrogallol was bactericidal. Cell number remained constant for treatment at MIC in the first 6 hours, followed by reduction in number. Higher

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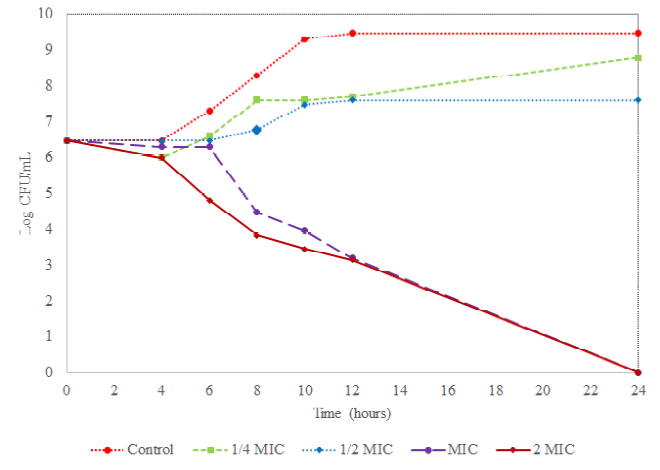

Figure 2 Time kill curve of various concentrations of pyrogallol against MRSA ATCC 33591

dosage of pyrogallol (2 MIC) would result in significant decrease in viability of MRSA and onset of detectable killing in shorter period of time. Reduction in cell number at higher dosage was noticed from t = 0 hour onwards (Fig 2).

Taguri et al.(6) reported that pyrogallol exhibited the strongest antibacterial activity among the selected polyphenols studied, namely epicatechin, catechol, epigallocatechin, caffeic acid, epigallocatechin-3-O-gallate etc. In addition, pyrogallol is active towards most of the Gram-positive and Gram-negative bacteria selected. This showed that it is a broad-spectrum antibacterial agent. Pyrogallol has also been reported could exhibit antibacterial activity against Pseudomonas putida, Pseudomonas pyocyanea, Corynebacterium xerosis (13). Interestingly, pyrogallol has also been reported could exert synergistic effect with Norfloxacin and Gentamicin in inhibiting S. aureus. The antimicrobial activity of these antibiotics together with pyrogallol against S aureus was enhanced, where the MIC was reduced up to 20-fold (9).

The number of pyrogallol rings is also well correlated to the antibacterial activity. Authors reported that compounds with pyrogallol group attached were likely to exhibit stronger antibacterial activity than those with catechol groups. For instance, prodelphinidins which carries a pyrogallol groups exhibited stronger antibacterial activity than procyanidins (6), although both compounds are structurally similar. Similar findings were also noticed in other polyphenol compounds: between gallic acid and protecatechuic acid, between myricitrin and rutin, and between pyrogallol and catechol. In addition, authors also reported that plant extracts which consisted of major compounds with pyrogallol groups showed greater antibacterial activity. On the other hand, extracts which consisted of phytochemicals (procyanidins or flavonoids) bearing the catechol and resorcinol groups as major constituents would exhibit weaker activity. These findings showed the importance of pyrogallol group in exhibiting stronger bioactivity than other similar hydroxyphenol groups.

There were studies which proposed the two possible antibacterial mechanisms: (1) oxidative stress, and (2) disruption on membrane fluidity. Pyrogallol has been reported to exhibit dual antioxidant/prooxidant properties (14, 15). The dual antioxidant/prooxidant property enable the compound to either scavenge or produce radicals depending on the environment (16). In this study, it is likely that the compound had generated reactive oxygen species, such as hydrogen peroxides (H2O2) and superoxide (15, 17) to inhibit the bacterial growth. Increase in H2O2 concentration was noticed in pyrogallol containing media (15). Lim et al(14) reported that pyrogallol inhibited the growth of Vibrio vulnifus, where the oxidative stress-related protein in bacteria was upregulated with the presence of pyrogallol. However, presence of antioxidants could reduce the inhibitory effect by pro-oxidants generated by pyrogallol.

Numerous studies have reported that polyphenol compounds targeted on bacteria cell membrane (18-21). Polyphenols was reported to mediate the antibacterial activity by adsorbing on to the surface of the bacterial cell wall before exhibiting the antibacterial activity (6). Literatures had reported that polyphenols could inhibit bacteria in three stages: (1) cell membrane attachment; (2) cell membrane fluidity modification; and (3) cell membrane structure disruption (1, 22-24). The binding of polyphenols to bacteria cell membrane could disrupt the membrane architecture. For instance, epicatechin gallate inhibits MRSA by insertion into the bacteria cytoplasmic membrane and disruption of penicillin-binding protein 2a-mediated ?-lactam resistance (22); berberine and piperine could interfere the microbial growth by intercalating the cell wall and DNA (23, 24); and tannins damaging the bacterial cell membranes of Listeria monocytogenes (25). Smith et al(26) reported that polyphenols could reduce the membrane integrity, inhibit oxidative phosphorylation and the cell transport processes. It is believed that pyrogallol could exhibit the lipid peroxidation towards MRSA cell membrane, where the free radicals extracted electrons from the bacterial cell membranes (27), modified the cell membrane fluidity and resulted in cell membrane disruption. Chedea et al(27)

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reported that bactericidal of polyphenols which exhibited pro-oxidation effect were likely to exhibit bactericidal effect by disrupting the ordered structure of phosphatidylcholine and phosphatidylethanolamine bilayer of the cell membrane. For instance, galloylated catechins which possessed higher phospholipid/water partition coefficients immersed in the phospholipid palisade intercalating within the hydrocarbon chains (28).

4. Conclusion

Our study demonstrated that pyrogallol exhibited strong antibacterial activity against MRSA. It exhibited bacteriostatic and bactericidal effect at 15.6 µg/mL. Time-kill kinetic assay showed that lower concentration of pyrogallol could slightly inhibit the exponential growth of MRSA, and higher concentrations were lethal to the bacteria. Possible bactericidal mechanism of pyrogallol was thoroughly discussed in this study. However, it is necessary to conduct more detailed studies on the mechanism of action.

Acknowledgement

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Conflict of Interest

All authors of this study declare that there are no conflicts of interest.

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Analysis of the Effectiveness of Drug Awareness Campaigns Using Google Trends

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Abstract

Globally, drug-related problems have attracted much attention from the public because of the negative health effects and the huge social burden. Therefore, Policymakers, healthcare institutions, and the people are concentrating on improving drug awareness to eradicate the abuse of illicit or prescription drugs for the destiny of a healthier community. They spent a lot to designate drug prevention campaigns as well as programs. However, the previous study has not measured the effectiveness of drug awareness campaigns comprehensively and accurately. The public was also understudied previously where the public learning preference and knowledge loophole of the drug are unclear yet. The foremost objective of this article is to figure out the effectiveness of drug awareness campaigns using Google Trends. It also aims at revealing audiences' preference of search method when they searching for the related information. This articleuses the qualitative method to explore the effectiveness of drug awareness campaign and the preferred search methods of the public to gain information about drugs by analyzing the data on Google Trends which tracks the public interest of "drugs" over time worldwide. The result found that the effect of the global drug awareness campaigns in 2018 is moderate and ephemeral and public prefers using the web search to collect information they want about "drugs". Globally, "pharmaceutical drugs"is the hottest topic related to drugs during the last year. This article finds a generally moderate influence of drug awareness campaigns in 2018. The public prefers to use Web Search to find information about drugs. Moreover, the top 5 countries where the "drugs" gains the highest attention from the public is different when the search method is different.

Key words : Drug awareness, Drug prevention campaigns, Effectiveness, Drug-related problems, Search preference, Google Trends.

1. Introduction

Globally, the drug has attracted much attention from the public because of the health problems it may cause. Substance abuse not only contributes to death but also relates to short-term or long-term health effects(Johnston, O'malley, Miech, Bachman, & Schulenberg, 2016). To treat dependence on the drug could cause a huge press to the whole society as well. However, the cruel situation is that the percentage of illegal drug users and drug abusers is shockingly high and the number is continuing to increase year by year based on the annual reports from the global drug-focused institution (UNODC, 2010)Drug deal even becomes a powerful source to stimulate world economic advancement.It also finds that most of these drug abusers did not get medical or mental treatment at all.

Therefore, Policymakers, healthcare institutions, and the people worldwide are concentrating on how to eradicate the abuse of illicit or prescription drugs for the destiny of a healthier community(Fonseca et al., 2017)They designate drug prevention campaigns as well as programsto, improve the awareness of the public towards the detrimental consequences of substance use. Drug awareness wasan essential concept in these campaigns which is characterized as the understanding and knowledge of nature, mechanism, signs, consequences, prevention methods etc. of substance use (Schmitt et al., 2011). It is a key element to prevent substance abuse because people who are more aware of drug use are less likely to misuse drug(Jordan & Andersen, 2017).

To improve drug awareness worldwide, the United Nations office sets June 26 as the World Drug Day when various campaigns and activities will be held to celebrate this date. They also decided an Action Week from June

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24 to June 30 worldwide. On World Drug Day 2018, Day of Action campaign was put into effect globally. And each country also made specific campaigns nationally. West, central and South Africa carried out "Listen First" campaign to raise drug awareness; India implemented a "Deep Dive" campaign to promote understanding and communication(UNODC, 2018); the United States executed the activity with the theme of publication; QuitStigmaNow was launched by Canada("World Drug Day 2018 - Vienna NGO Committee on Narcotic Drugs," 2018).

The previous research has systematically explored the possible factors which may cause individuals to use the substance. O'Hara, Armelie, and Tennen (2015) found the role of people's intention and social circle in this process(O'Hara, Armeli, & Tennen, 2015). Yang and Xia (2019) found that the lack of knowledge towards the possible harmful impact and the lack of punishment towards drug abuse are the two main reasons for drug misuse(Yang & Xia, 2019). It was argued that drug use is initially a kind of social activity but ends with social isolation (Tam, Kwok, Lo, Lam, & Lee, 2018). Therefore, based on these studies, drug prevention programs are carried out to improve public awareness.

However, drug awareness campaigns are not easy to implement because they consume a large amount of money and human resources to set up the whole plan(Substance Abuse and Mental Health Services Administration, 2014). Several crucial features of effective programs are identified, including highlighting the harmful effect, offering information on how to resist temptation, and targeting audiences sharply (Botvin & Griffin, 2007).

While the prior study has only analyzed the effectiveness of these campaigns by systematic reviews (Das, Salam, Arshad, Finkelstein, & Bhutta, 2016). It has not measured the effectiveness of more comprehensively and accurately.The public was also understudied previously where the public learning preference and knowledge loophole of the drug are unclear yet. As drug misuse is a global issue, it has not been studied from a global level but only was targeted from the specific country.

Currently, with the advancement of communication technology, audiences tend to prefer search information on the internet through websites because of its simple operation and abundant resources (Chie et al., 2015). This trend can be used to test the awareness of audiences towards the drug. Google Trendsis a useful instrument which can track the search frequency of certain topics around the world through different search methods, including web search, image search, news search, google shopping, and YouTube search. Choi and Varian (2012) also claimed that Google Trends is useful to help predict trends(CHOI & VARIAN, 2012). Thus, drug awareness can be measured globally by the public's interest in "drugs" on the search engine.

This article would come up with the following questions:

1. Is there a difference in public drug awareness before and after the drug awareness campaignsglobally in 2018?
2. What are the preferred search methods for gaining information about drug issues during the last year globally?
3. What are the top countries where drug awareness is relatively higher during the last year?
4. What related topics and queries appear the most frequently worldwide during the last year?

The foremost objective of this article is to figure out the effectiveness of drug awareness campaigns using Google Trends. It also aims at revealing audiences' preference of search method when they searching for the related information. In addition, the article plans to find out the countries with relatively higher drug awareness and the top hot topics and queries related to this topic worldwide.

The article tries to investigate some practical implications for drug awareness campaigners to analyze the quality of the campaigns and to draw more effective plans. Although the drug misuse issue is intricate and prominent, the campaigners and researchers are still dedicating to find the most viable way to improve public drug awareness. Knowing the effectiveness of drug awareness campaigns can also assist drug campaigners to learn fromprevious experiences for the purpose of the advancement of the next program. Reflecting the past campaigns whether they are successful or not can reduce or eliminate the risk of failure considering the difficulty of setting up a campaign. The preferred search method of the public can suggest the correct media platforms and content forms for the content producers to target on, which can help increase the circulation and traffic of the content. Therefore, it can reach a wider public to exert the best power. The hottest related topics and queries can indicate

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the weakness of the drug awareness campaign and education programs as well as point out the future direction for campaigners to produce personalized content. Knowing the audiences better can help optimize the content. The comparison between countries globally could indicate the strength of some countries so that the rest can learn from the success or evade from the failure of these top countries. It would help solve drug issues from a global level and as a team.

2. Materials and Methods

This article will use the qualitative method to explore the effectiveness of drug awareness campaign and the preferred search methods of the public to gain information about drugs by analyzing the data on Google Trends which tracks the public interest of "drugs" over time worldwide. Because the first purpose of this article is to analyze the effectiveness of the drug awareness campaign in 2018, the sample data will be drawn from Google Trends during the last year, including the date of World Drug Day - June 26, 2018, and the Action Week (June 24 to June 30). The location will be chosen as worldwide. All categories will be selected. Each set of results from web search, image search, news search, google shopping, and YouTube search will be selected as the sample to detect whether there is any difference in the public interest of "drugs" before and after the drug awareness campaigns in 2018.

As the second objective of this research is to find out the preferred search methods during last year globally, the level of public interest on each search methods will be compared. The term is still "drugs". The location is worldwide. The time period is between the past 12 months. And all categories will be contained.

In order to understand the topic more comprehensively, the data concerning the interest by region of "drugs", related topics and queries will be collected under all these five search platforms. The keyword, location, time frame, and categories will still be the same.

Table 1: Search Strategy

Search term	Drugs
Location	Worldwide
Time frame	Past 12 months (March 19, 2018 – March 19, 2019)
Categories	All categories
Search methods	Web search, image search, news search, google shopping, YouTube search
Interest by region	Include low search volume regions (region areas)
Related topics and queries	Top

3. Results and Discussion

The Effectiveness of Drug Awareness Campaign Worldwide

Figure 1 observes a stable and high-interest level of "drugs" after June 26, 2018. During the whole year, the interest level falls between 75 and 100. And it reports no difference before and after the drug awareness campaign in 2018 globally.

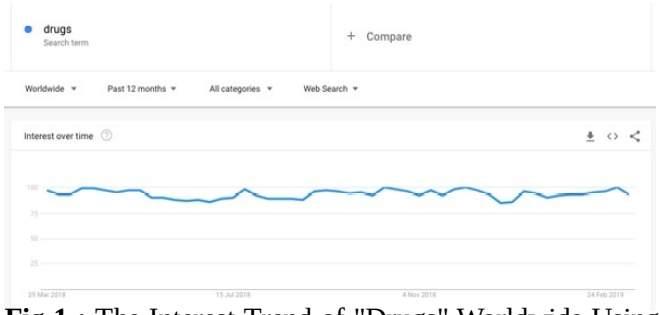

Fig 1 : The Interest Trend of "Drugs" Worldwide Using Web Search

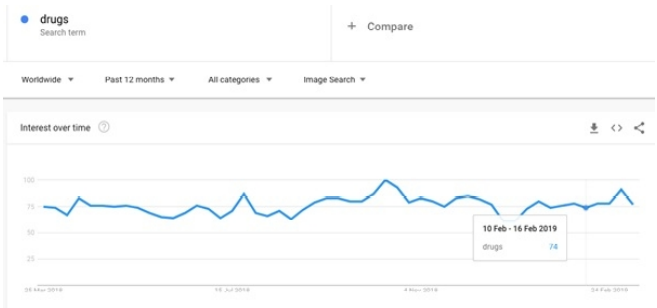

Fig 2 : The Interest Trend of "Drugs" Worldwide Using Image Search

From Figure 2, a slight rise in the interest level of "drugs" is indicated during the period of the global drug awareness campaigns. However, the impact of the campaigns is not striking. According to Figure 3, public records a short period of moderately growing focus on "drugs" from June 24 to June 30. Based on Figure 4, a

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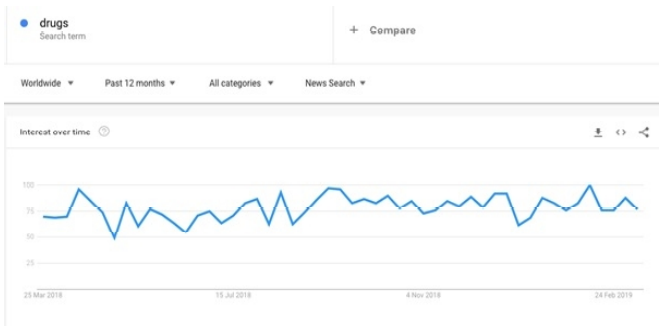

Fig 3 : The Interest Trend of "Drugs" Worldwide Using News Search

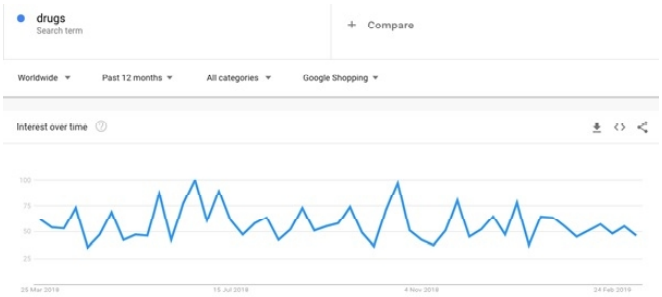

Fig 4 : The Interest Trend of "Drugs" Worldwide Using Google Shopping

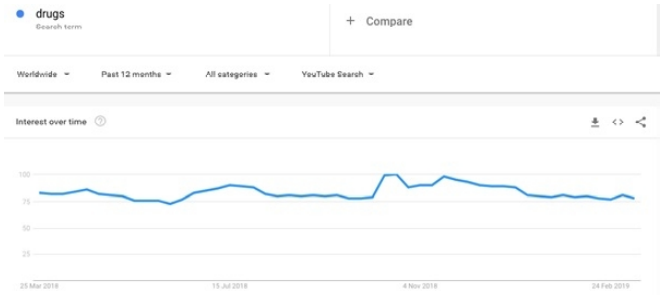

Fig 5 : The Interest Trend of "Drugs" Worldwide Using YouTube Search

sharp increase is witnessed from June 24 to June 30. While Figure 5 shows a continuously and slightly increasing public interest level of "drugs".

Preferred search methods

By comparing the above five figures, it can be concluded that web search is the most often used method to find information about "drugs". The interest level of "drugs" using web search is nearly 100 over the whole year. While the interest levels using other search methods are comparatively lower.

Top drug awareness countries

From the following figures, it is clear that the top 5 countries interested in "drugs" topic are Nigeria, Liberia, Zambia, Ghana, and Tonga for Web Search, Tonga, Dominica, American Samoa, Fiji, and St. Kitts & Nevis for Image Search, Antigua & Barbuda, Cayman Islands,

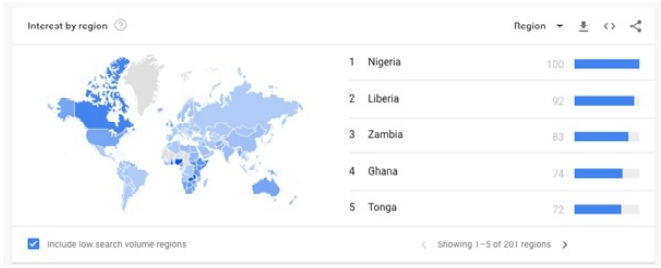

Fig 6 : Top 5 Regions Using Web Search

Fig7 : Top 5 Regions Using Image Search

Fig 8 : Top 5 Regions Using News Search

Fig 9 : Top 5 Regions Using Google Shopping

Fig 10 : Top 5 Regions Using YouTube Search

Grenada, Jersey, and Bermuda for News Search, Canada, Philippines, Ireland, Singapore, and India for Google Shopping, Guernsey, Anguilla, Isle of Man, Jersey, and Netherlands for YouTube Search.

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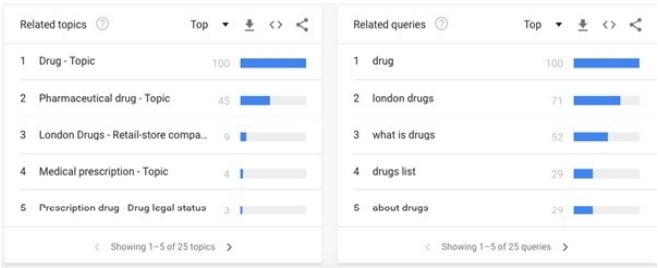

Fig11 : Top Related Topics and Related Queries Using Web Search

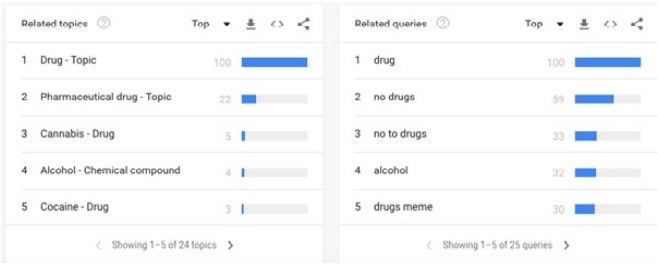

Fig 12 : Top Related Topics and Related Queries Using Image Search

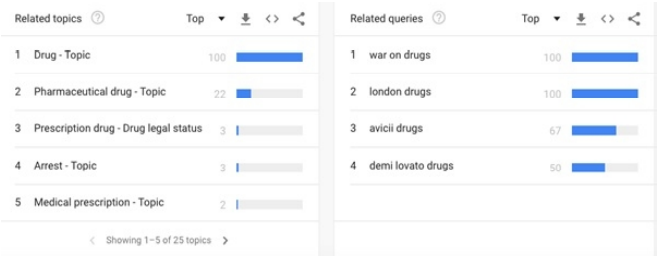

Fig 13 : Top Related Topics and Related Queries Using News Search

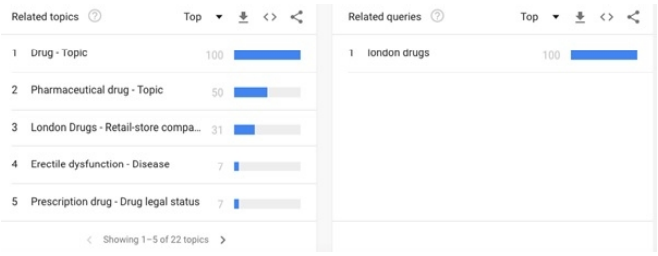

Fig 14 : Top Related Topics and Related Queries Using Google Shopping

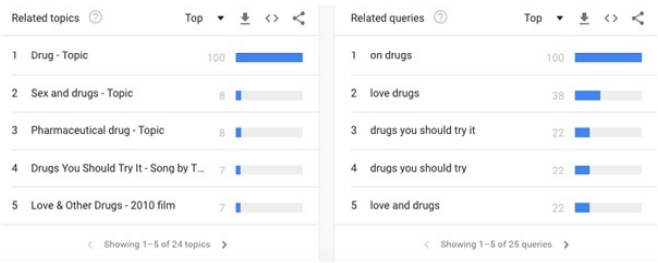

Fig 15 : Top Related Topics and Related Queries Using YouTube Search

Top related topics & related queries

The following five figures illustrated the top related topics and queries by using five search methods. Figure 11 shows that by Web Search, the top related topics are "Drug - Topic" and "Pharmaceutical drug - Topic"; the top related queries are "drug", "London drugs", and "what is a drug". It can be seen from Figure 12 that "Drug - Topic" and "Pharmaceutical drug - Topic" are the top related topics; "drug" and "no drugs" are the top related queries by using Image Search. Based on Figure 13, the top related topics by using News Search are still the same from using Web Search and Image Search. The top related queries are "war on drugs", "London drugs", "avicii drugs", and "demi lovato drugs". Based on Figure 14, the top related topics are the same as the above results. While the top related queries are different. "London drugs" is the only top query using Google Shopping. According to Figure 15, the top three related topics are "Drug - Topic", "Sex and drugs - Topic", "Pharmaceutical drug - Topic" using YouTube Search. And the top related queries are "on drugs" and "love drugs".

Generally, the effect of globaldrug awareness campaigns in 2018 is moderate and ephemeral. After the campaign, the interest in "drugs" only improved a little. This little impact fades away and the interest level drops down immediately after the Action Week. Weiss and Tschirhart (1994) also draw a similar conclusion that drug campaigns produce a "boomerang" influence on public awareness where the effect lasts shortly(Weiss & Tschirhart, 1994). They found that the impact only existed during the campaign while the situation came back to the original point after the campaign. A survey conducted in French received a similar result that generally, the campaign increased recipients' drug awareness partially and inconsistently (Cuchet-Chosseler, Bocoum, Camara, Abad, & Yamani, 2011). The campaign is mildly effective and lasts for a short period.

The result also finds that the public prefers using the web search to collect information they want about "drugs". The hotness of "drugs" was highest on the web search among these five search tools. This is consistent with the previous research where the web search gains more and more attention from the public (Chuklin, Markov, & Rijke, 2015). They also indicate that people around the world have a tendency to find information by using web search. One advantage of web search is that all the relevant contents are available on this instrument including images, videos, news, and links to shopping products (Xie et al., 2017). That means all the information

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from the rest of the search methods can be found on web search results plus other forms of information. Therefore, it can be concluded that users will gain more comprehensive results from a web search.

When the search method is different, the top 5 counties with the highest interest in "drugs" are totally different. The difference in searching preference in different countries may contribute to this result. People in different countries may prefer different search methods in order to satisfy different information needs.

As for the top related topic with "drugs", "pharmaceutical drugs" appears most often under these five search methods. In this area, "pharmaceutical drugs" gain the most attention from the public. Thus it becomes the top related topic. According to (Hulme, Bright, & Nielsen, 2018), pharmaceutical drugs non-medical use which including the prescription or over-the-counter drug is much more prevalent than illicit drugs globally(Hulme, Bright, & Nielsen, 2018). Pharmaceutical drugs have become a public concern with their high costs on health, society, and economics (Saha et al., 2016). Moreover, social media also increase the public's exposure to misleading or dangerous information about pharmaceutical drugs through pharmaceutical companies' marketing (Tyrawski & DeAndrea, 2015). Therefore, "pharmaceutical drugs" becomes the hottest topic concerning drugs which receives the highest interest from the public.

4. Conclusion

Generally, the top related topics are "Drug - Topic" and "Pharmaceutical drugs - Topic" among these five different search methods. The top related queries are "drug", "London drugs", and "war on drugs". The finding in the article is significant to guide the way for future drug awareness campaigns. It can give practical solutions for content producers and health professionals to target the drug problem.

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Current Trends in Biotechnology and Pharmacy 188
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Sun Protection Effect of 2-Hydroxy-4-(Octyloxy) Benzophenone in Sunscreen Creams Formulations by a Combination of Inorganic UV Filters

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Abstract

Overexposure of ultraviolet (UV) radiation, especially UVB (280-320 nm) and UVC (200-280 nm) have a harmful effect on the skin. Sunscreen such as derivatives of benzophenone can protect the skin from these detrimental effects. In this research, we evaluated the potency of 2-hydroxy-4-(octyloxy) benzophenone as a sunscreen and improved the ability by combining it with the physical blocker TiO_2 or ZnO in the form of a cream formulation. We use a D-optimal mixture design to obtain the cream formulation with high Sun Protection Factors (SPF) and acceptable characteristics. Several cream formulations containing 2-hydroxy-4-(octyloxy) benzophenone and TiO_2 or 2-hydroxy-4-(octyloxy) benzophenone and ZnO were prepared with concentrations of 5-10%. The creams were tested for the physicochemical parameters such as pH, color, odor, homogeneity, viscosity, and stability. The SPF was observed by spectrophotometry and the value was calculated using the Mansur equation. SPF value of 2-hydroxy-4-(octyloxy)benzophenone, TiO_2, and ZnO were 25.21±0.47; 24.74±0.35; 3.20±0.05, respectively. SPF value of creams combining 2-hydroxy-4-(octyloxy) benzophenone and TiO_2 were in the range of 4.140-6.326. Furthermore, the SPF value of creams combining 2-hydroxy-4-(octyloxy) benzophenone and ZnO were in the range of 3.609-8.052. The creams meet the requirement of physicochemical properties with acceptable characteristics. They were stable when the creams kept at room temperature for one month. In this current study, the formulation of sunscreen creams with high SPF and acceptable characteristics obtained by a combination of 10% 2-hydroxy-4- (octyloxy) benzophenone and 5% titanium dioxide or ZnO.

Key words : 2-Hydroxy-4-(octyloxy) benzophenone . Sunscreencream.Sun protection factor. TiO_2.ZnO

1. Introduction

Exposure of solar ultraviolet (UV) radiation in excessive conditions can cause skin problems including sunburn, edema, erythema, immune suppression, wrinkles, dermatitis, urticarial, aging, hypopigmentation, hyperpigmentation, and skin cancer [1-2]. The ozone layer can absorb 100% UVC (200-290 nm), 90% UVB (290-320 nm), and a little amount of UVA (320-400 nm). However, depletion of the ozone layer causes increased UV transmission to the earth's surface. Sunscreen is used to reduce skin damage from UV radiation. Sunscreen is offered in many formulas like gel, lotion, stick, cream, lip balm, and spray. Gel preparations are commonly used for sunscreens because the gel provides some advantages including a cooling effect on the skin, elastic, and transparent appearance.

Based on the basis of its mechanism of action, there are 2 types of sunscreens including chemical absorbers (organic) which absorb the UV radiation and physical blockers (inorganic) which reflect and scatter UV radiation [3-4]. Previously, sunscreen only focuses to cover UVB radiation. Currently, the FDA recommends the use of broad-spectrum sunscreens which cover not only the entire spectrum of UVB but also the UVA. Zinc oxide, a physical blocker sunscreen, was able to protect skin from UVA radiation [5]. However, physical blocker is less proficient against UVB protection [6]. A combination of sunscreen that can protect the skin not only in the UVB region but also in the UVA regions, will provide added value.

The benzophenones derivative, 2-hydroxy-4-(octyloxy) benzophenone (HOB) is commercially available. The compound has been used extensively in organic synthesis. Based on the structure, we considered the molecule to be applicable as a UV filter. Furthermore, the compound was prepared to develop sunscreen cream

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formulations with satisfactory characteristics. The purpose of this study was to evaluate in-vitro sunscreen activity of a cream formulation containing HOB and improved the ability by combining it with the physical blocker TiO_2 or ZnO.

2. Materials and Methods

Materials **:** Zinc oxide was from KOBO. 2-hydroxy-4-(octyloxy)benzophenone was obtained from Sigma-Aldrich. Methylparaben, propylparaben, mineral oil, propylene glycol, triethanolamine, glycerin, ZnO, and aquadest were obtained from Brataco Chemica (Indonesia). Cetyl alcohol, glyceryl monostearate were purchased from Cognis. Olive oil and ascorbic acid were from Prima Chemical.

Optimization of cream formulation **:** The experimental design was a two factor two-level general factorial (Design Expert 7.0.0) and seven formulations were prepared (Table 1). The amount of HOB (X1) and TiO_2 or ZnO (X2) were selected as independent variables. The number of independent variables was optimized for dependent variables: viscosity and SPF. The low (5%), and high (10%) are the values of HOB, TiO_2 or ZnO, Mathematical equations were generated for each parameter. The mathematical models were studied for significance.

Table 1 : Composition of cream formulations with different amount of 2-hydroxy-4-(octyloxy) benzophenone, TiO2, and ZnO

Ingredients	Run1	Run2	Run3	Run4	Run5	Run6	Run7	Negative Control
Ingredients A								
Cetyl alcohol (g)	1	1	1	1	1	1	1	1
Mineral oil (g)	10	10	10	10	10	10	10	10
Olive oil (g)	10	10	10	10	10	10	10	10
Propylparaben (g)	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Glyceryl monostearate(g)	16	16	16	16	16	16	16	16
Ingredients B	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Methylparaben (g)	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Glycerin(g)	7	7	7	7	7	7	7	7
Ascorbic acid	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
HOB	5	10	10	6.25	8.75	7.5	5	0
TiO_2/ZnO	10	5	5	8.75	6.25	7.5	10	0
Aquadest ad (ml) qs ad to	100	100	100	100	100	100	100	100

Cream formulation **:** The basic sunscreen formulation was prepared according to the formula recommended by EIRI with some modifications [7]. The Ingredients B (methylparaben, glycerin) were first heated (70ºC) as well as ingredients A (glyceryl monostearate, cetyl alcohol, mineral oil, and propylparaben). The ingredients A were poured into a warm porcelain mortar and a required quantity of olive oil was added to the mixture. The water phase (B) was added slowly to the oil phase (A). The ascorbic acid was added and grinded well. After that, ZnO or TiO_2 was dissolved in hot distilled water and added to the mixture. Finally, HOB was added to form a homogeneous cream.

Organoleptic evaluation **:** All creams were observed for physical form including texture, color, and odor. These physical characteristics were observed by visual tested.

Physicochemical evaluation **:** Physicochemical evaluation of cream was tested for homogeneity, pH, viscosity, and spreadable. Homogeneity was observed by applying the cream on a glass object. The cream must give indicate of a non-grained appearance and no visible spot. The pH was measured using a pH meter. Viscosity was measured in Brookfield viscometer (DV-1 Prime) using a LV-4 spindles and a rotation rate of 60 RPM. Finally, the spreadable test of the cream was measured by applying the cream (500 mg) between two pieces of circle glass plates (diameter 15 cm) for one-minute compression. After that, the standard weight (50 g) was applied to the upper plate. After one minute's compression, the spreading diameter of the cream was measured. All measurements were made in triplicate.

Determination of SPF value of HOB, TiO_2, ZnO, and cream formulation **:** Samples were prepared according

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to the method recommended by Dutra [8]. One gram of sample was weighed and diluted to 100 ml with ethanol. The solution was ten milliliters. Afterwards 5.0 ml of filtered solution was transferred to a 50 mL volumetric flask, adjusted with ethanol. The absorbance of the final solution was measured by spectrophotometry in the range of 290 to 320 nm for every 5 nm wavelength interval. Finally, the SPF value was calculated using the Mansur equation [9].

2. Results and Discussion

Today, many strategies are proposed to obtain sunscreens with high SPF values and can cover a broad spectrum of UV radiation. One strategy is to combine UVA and UVB filters as well as chemical and physical sunscreens. This sunscreen combination method tends to be preferred because it only requires a preparation process that is identical to the conventional one, and only the required formulation ingredients are adjusted. In this study, we used ZnO or TiO_2 as a physical sunscreen and HOB as a chemical sunscreen.

The previous study in our research group show that the HOB has a higher SPF value of 25.2±0.5 compared with benzophenone-3,3',4,4'-tetracarboxylate dianhydride (6.4±0.2) or 2-benzoylbenzoic acid (2.7±0.4) [10]. The SPH value was also higher than that of SPF of natural sunscreen corn cob (4.95±0.86) [11]. The absorbance of HOB, ZnO, and TiO_2 at UV region (200-400 nm) were recorded and shown in Figure 1. It presented that the absorbance of HOB in the UVB region was higher than that of UVA and part of UVC. The absorbance of TiO_2 in the UVA and UVC region were higher than that of HOB. On the other hand, ZnO has relatively similar absorbance throughout the UV region, and also ZnO has a greater absorbance in the UVA region than that of HOB. The SPF value of HOB, TiO2, and ZnO were 25.2±0.5; 24.7±0.3; and 3.2±0.05 which is considered ultra, ultra, and extra protection, respectively, in terms of sunscreen protection.

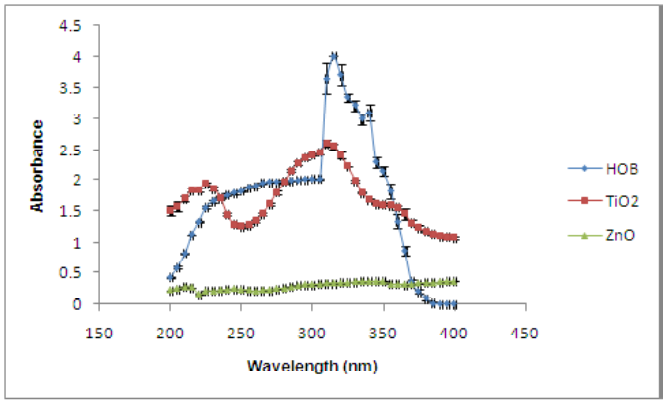

Fig. 1. Absorption curve of the solution of 2-hydroxy-4-(octyloxy)benzophenone, ZnO, and TiO2 (right). The concentration of 1% in ethanol.

Table 2 : Physicochemical parameter evaluation of HOB-TiO2 creams

Formulation	Color	Odor	pH	Homogeneity	Viscosity (cps)	Spreadability (cm)
Negative control	White	Characteristic of olive oil	4.01±0.01	Homogenous	9136.66±106.92	4.66±0.07
Run1	White	Characteristic of olive oil	5.95±0.02	Homogenous	9546.66±94.51	4.65±0.10
Run2	White	Characteristic of olive oil	5.90±0.02	Homogenous	7853.33±96.09	4.21±0.11
Run3	White	Characteristic of olive oil	6.56±0.02	Homogenous	8236.66±105.98	4.65±0.06
Run4	White	Characteristic of olive oil	5.58±0.03	Homogenous	8956.66±80.82	4.24±0.10
Run5	White	Characteristic of olive oil	5.09±0.03	Homogenous	8426.66±96.09	4.91±0.06
Run6	White	Characteristic of olive oil	5.86±0.03	Homogenous	8466.66±96.09	4.91±0.05
Run7	White	Characteristic of olive oil	6.19±0.02	Homogenous	9756.66±105.98	4.30±0.01

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Table 3 : Physicochemical parameter evaluation of HOB-ZnO creams

Formulation	Color	Odor	pH	Homogeneity	Viscosity (cps)	Spreadability (cm)
Negative control	White	Characteristic of olive oil	4.01±0.01	Homogenous	9136.66±106.92	4.66±0.07
Run1	White	Characteristic of olive oil	7.04±0.020	Homogenous	8823.33±76.37	4.24±0.07
Run2	White	Characteristic of olive oil	7.02±0.015	Homogenous	8783.33±65.06	4.22±0.09
Run3	White	Characteristic of olive oil	7.00±0.015	Homogenous	8636.66±55.07	4.25±0.04
Run4	White	Characteristic of olive oil	6.92±0.030	Homogenous	8323.33±100.16	4.19±0.05
Run5	White	Characteristic of olive oil	7.10±0.023	Homogenous	7870±62.44	4.10±0.07
Run6	White	Characteristic of olive oil	7.08±0.025	Homogenous	8143.33±25.16	4.13±0.08
Run7	White	Characteristic of olive oil	7.05±0.011	Homogenous	8696.66±115.90	4.20±0.07

The formulated sunscreen creams were evaluated for several physicochemical tests and the results were displayed in Table 2-3. The formulate creams showed good acceptable odor, characteristic odor of olive oil, and were white color. The viscosity of formulated sunscreen creams ranged from 7853 to 9546 cps. The concentration of TiO_2 has a positive effect on the viscosity. It can be concluded that the creams have good viscosity (2.000-50.000 cps, SNI). Viscosity is an important parameter for evaluating cream preparations and the characteristics are related to the spreadable of the creams.

The pH value of formulated sunscreen creams combination of HOB and TiO_2 ranged from 5.09 to 6.56. Whereas the pH value of formulated sunscreen creams combination of HOB and ZnO ranged from 6,92 to 7.08. Cream combination of HOB and TiO_2 has a lower pH value than ZnO. All of formulated sunscreen creams meet the requirements for sunscreen preparation ranging from 4.5-8.0 [12]. The creams would not irritate if applied to the skin.

The SPF values of the sunscreen creams containing both HOB and TiO_2/ZnO in various concentrations were displayed in Table 4. The results show that the SPF values in the UVB region were directly dependent on HOB or TiO_2/ZnO concentrations. The creams have a low SPF values compare with the active compounds, this is due to their low amount of active compounds.

Table 4 : SPF of HOB-TiO_2 and HOB-ZnO cream formulations

Formulation	SPF	
	Combination of HOB and TiO_2	Combination of HOB and ZnO
Negative control	0.580±0.005	0.498±0.014
Run1	4.872±0.018	4.888±0.067
Run2	6.326±0.013	8.052±0.022
Run3	6.164±0.017	7.606±0.018
Run4	4.140±0.011	3.610±0.009
Run5	6.389±0.018	5.694±0.037
Run6	5.063±0.011	6.419±0.022
Run7	4.489±0.018	4.531±0.034

To optimize the creams for viscosity and SPF value, a general factorial method is applied in this study. The amount of HOB and TiO_2/ZnO was chosen as independent variables. A statistical model was used to observe the responses.

The ANOVA (analysis of variance) of HOB-TiO_2 creams displayed that the viscosity was appropriate to explain the main effect model (P-value 0.0024) as well as SPF (P-value 0.0408). By developing a normal probability plot of internally studentized residuals, a patterned was made for the normality statement (Fig. 2).

Furthermore, the ANOVA of HOB-ZnO creams showed that the viscosity was appropriate to explain the main effect model (P-value 0.0294) as well as SPF (P-

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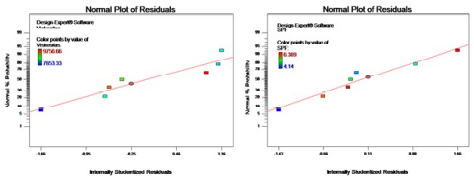

Fig. 2. Plot of internally studentized residuals vs. predicted response of viscosity (left) and SPF (right) of HOB-TiO_2 creams.

value 0.0368). A normal probability plot of internally studentized residuals was displayed in Fig 3. The statement of normality is fulfilled when the residual plot is approached along a straight line. The plot is acceptable, so we conclude that the resulting general factorial equation can be used in predicting SPF or viscosity of HOB-TiO_2 creams or HOB-ZnO creams.

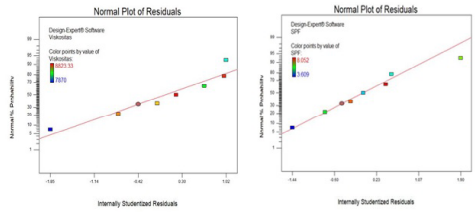

Fig. 3. Plot of internally studentized residuals vs. predicted response of viscosity (left) and SPF (right) of HOB-ZnO creams.

The fitted equation relating the responses viscosity to the transformed factors of HOB-TiO_2 creams was shown in equation 1 and Fig 4. The equation shows a positive interaction with HOB of 8082.88 and TiO_2 of 9628.80. It means that HOB and TiO_2 will increase viscosity, and TiO_2 has the greatest effect on increasing viscosity compare with HOB. In addition, there is a negative interaction with the combination of HOB and TiO_2 with an interaction coefficient of -1196.14. Furthermore, the fitted equation relating the responses SPF to the transformed factors is shown in equation 2 and Fig 4.

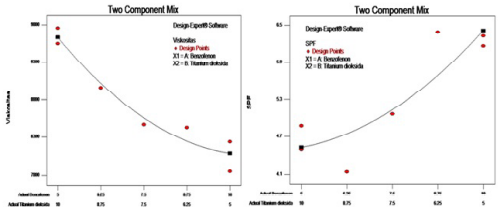

Fig. 4. The graph relates to the response of viscosity (left) and SPF (right) to the transformed factors of HOB-TiO_2 creams.

$$Y = 8082{,}88A + 9628{,}80B - 1196{,}14AB \quad (1)$$

Y = viscosity
A = Concentration of HOB
B = Concentration of TiO_2

$$Y = 6{,}41A + 4{,}52B - 1{,}34AB \quad (2)$$

Y = SPF
A = Concentration of HOB
B = Concentration of TiO_2

The same way was used to observe the responses of viscosity and SPF to the transformed factors of HOB-ZnO creams. The results were shown in equation 3-4, and Fig 5.

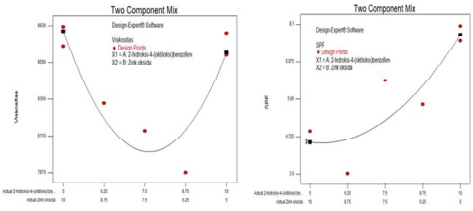

Fig. 5. The graph relates to the response of viscosity (left) and SPF (right) to the transformed factors of HOB-ZnOcreams.

$$Y = 1{,}24A + 0{,}59B - 0{,}15AB \quad (3)$$

Y = viscosity
A = Concentration of HOB
B = Concentration of ZnO

$$Y = 1{,}24A + 0{,}59B - 0{,}15AB \quad (4)$$

Y = SPF
A = Concentration of HOB
B = Concentration of ZnO

The desirability value was used to find out the best viscosity and SPF of 7 runs. The optimized of HOB-TiO_2 cream formulation obtained by using design experts was a combination of 10% of HOB and 5% of TiO_2, with viscosity of 8083 cps, SPF 6.4, and desirability of 1. Furthermore, the optimized of HOB-ZnO cream formulation was a combination of 10% of HOB and 5% of ZnO, with viscosity and SPF of 8651 cps and 7.8, respectively. The desirability obtained by the software was 0.940.

4. Conclusion

The 2-hydroxy-4-(octyloxy) benzophenone, TiO_2, and ZnO were exhibited sun protection activity. The benzophenone combined with TiO_2 or ZnO can be formulated as a cream with satisfying physical characteristics. In this current study, the formulation of sunscreen creams with high SPF and acceptable characteristics obtained by a combination of 10% 2-hydroxy-4-(octyloxy)benzophenone and 5% TiO_2 or ZnO.

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Conflict of Interest

The Authors declare that they have no conflicts of interest.

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In Silico Studies of Green Tea Catechins Against HER-2 Receptor in Breast Cancer

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Abstract

Green tea catechins have been widely studied and known to have anticancer activity, including breast cancer. Breast cancer is cancer with the highest prevalence in Indonesia, after cervical cancer. HER-2 (Human Epidermal Growth Factor Receptor-2) has a crucial role in the development of breast cancer. Thus, this protein is widely used as a therapeutic target. In this study, catechin activities on HER-2 Receptor Tyrosine Kinase (RTK) domain of breast cancer are investigated through in silico study. Four catechin compounds, namely EGCG, EGC, ECG, EC, and native ligand, were given docking and molecular dynamic simulations. Molecular docking is used to study the interaction of protein-ligand using AutodockTools. The stability of amino acid residue interaction with catechin was identified through molecular dynamics using GROMACS and the binding free energy was calculated using MM-PBSA. Among the four catechin compounds, EGCG has the best RMSD value. This indicates that EGCG has the best structural stability. The value of binding free energy (ΔG) of catechin compounds is greater than the native ligand, showing that the compounds have a lower affinity for HER-2. The result reveals that catechin compounds have lower activity than the native ligand. However, catechin compounds have the same active site, and three catechin compounds can also interact with ASP863 which is an important residue in HER-2. Therefore, catechin compounds, especially EGCG, EGC, and ECG are potential to be developed into HR-2 inhibitors through structural modification.

Key words : Catechin. Molecular docking. Molecular dynamics. HER-2. Breast cancer.

1. Introduction

Breast cancer is cancer commonly suffered by women. Based on IARC (International Agency for Research on Cancer) data in 2018, breast cancer is the most common type of cancer in Indonesia. In the development of breast cancer, there are some protein roles. One of the proteins is HER-2. HER-2 is a transmembrane receptor tyrosine kinase that activates multiple proliferative signaling, including PI3K/ Akt and Ras/ MAPK(1). In normal cells, HER-2 has a role in cells' growth and proliferation. In the case of HER-2 positive breast cancer, the excessive HER-2 expression causes the increase of cancer cell activity, and the tumor which grows faster, is more aggressive, is less sensitive to hormone therapy, and chemotherapy (2).

HER-2 has been widely studied as a therapeutic target for HER-2 positive breast cancer. In HER-2 positive breast cancer, the amount of HER-2 overexpresses around 20-30%. Recently, most patients with advanced HER-2 positive breast cancer do not recover from their illness. They, on the contrary, getresistance to therapeutic agents that target HER-2 (3).

There are many in vitro and in vivo researches showing the correlation between consuming green tea and a reduced risk of breast cancer (4)(5). The ability of green tea to protect against breast cancer seems to be mediated by catechins, which are polyphenol (6)(7). The greatest compounds of catechin is epigallocatechin-3-gallate (EGCG), epigallocatechin (EGC), epicatechin-3-gallate(ECG), and epicatechin (EC) (8).

Catechins have been known to have many therapeutic activities, including anticancer activities(9). Green tea catechins have been proven to inhibit breast cancer cell proliferation and block carcinogenesis (10). In this research, the activity of catechins against HER-2 Tyrosine Kinase domain of breast cancer is investigated through in silico study.

2. Materials and Methods

Materials: The crystal structure kinase domain of HER-2(human epidermal growth factor receptor 2) receptor were obtained from protein data bank (PDB code: 3PP0)

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(11). Catechin structures (EGCG, EGC, ECG, EC) were made using ChemDraw Ultra 8.0, and Chem3D Ultra 8.0software.

The used software was AutoDock 4.2.6.and AutoDockTools 1.5.6 (The Scripps Study Institute, downloaded in http://www.autodock.scripps.edu/), BIOVIA Discovery Studio 2017 (http://www.accelrys.com/u), Gromacs v.5.1.1 (www.gromacs.org), ChemDraw Ultra 8.0 (https://chemistry.com.pk/software/free-download-chemdraw-ultra-8/), Gaussian 09 (https://gaussian.com).

Protein and ligand preparation **:** The crystal structures of HER-2 kinase domain obtained from protein data bank (12)(3PP0) were in the complex form and native ligand. The protein was separated from the ligand, was removed all its water molecules and was added with hydrogen atom. In this research, catechins (EGCG, EGC, ECG, EC) were used as the ligand. The ligand structures (2D and 3D) were made using ChemDraw Ultra 8.0, then were determined their physicochemical parameters. The 3D ligand structure was optimized through Gaussian using DFT/ B3LYP (13) with a STO-3G basis set to reach its stationary points.

Molecular docking **:** Molecular docking was conducted through AutoDockTools v.4.2.3. Software. The validation of molecular docking was conducted by re-docking the native ligand (14). Crystal structures of HER-2 kinase domain have 2-{2-[4-({5-chloro-6-[3- (trifluoromethyl) phenoxy] pyridin-3-yl} amino) -5H-pyrrolo[3,2-d]pyrimidin-5-yl] ethoxy} ethanol as the native ligand (11) and the surrounding residues, which are defined as the active site with geometric position (X = 16.38, Y = 17.39, Z = 26.21 Å). The pose with the lowest binding energy, obtained from the docking result, was then chosen for MD simulation.

Molecular dynamics simulation **:** All MD simulations were carried out using GROMACS v.2016.3 package with AMBER 99SB force field (15). The partial charges and topology files of ligands were produced by ACPYPE, a tool based on ANTECHAMBER, with the intermolecular potential represented as a sum of Lennard-Jones (LJ) force and pairwise Coulomb interaction, electrostatic force was determined by the particle mesh Ewald (PME) method. Initial atomic velocities were created on the basis of Maxwellian distribution at the absolute temperature of 310 oK. Numerical integrations were calculated by the velocity Verlet algorithm. The system was solvated in a cubic box with TIP3P water model, and then sufficient chlorine and sodium atoms were added to neutralize the system. The next step is equilibration to make the whole system at constant temperature and pressure. All MD simulations were conducted for 10 ns. The trajectories were analyzed and visualized using Discovery studio.

3. Results and Discussion

The Human Epidermal Growth Factor Receptor type 2 (HER-2) is a member of the oncogenic proteins family, which is the main target of cancer therapy including breast cancer (16). The structure of tyrosine kinase of HER-2 can be seen in Fig. 1. In this research, the four catechin compounds of Camellia sinensis were docked into binding pocket of HER-2 as their native ligands.

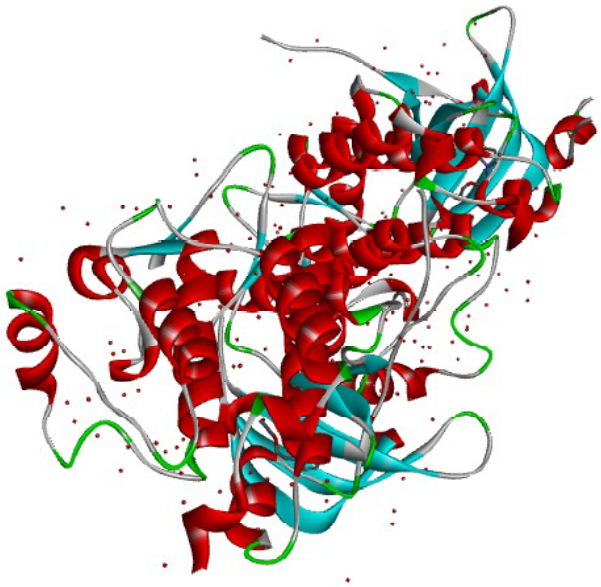

Fig. 1. The crystal structure kinase domain of HER-2

Molecular docking

Validation of the docking process was conducted through re-docking method using AutoDock. The validation was conducted in the active site of the native ligand on crystallographic results. The re-docking result indicates RMSD value of 1.19 Å, meaning that the atom position inside the ligand of the re-docking result is not much different from the position of crystallographic ligand (14). This result indicates that this method can be used for docking process.

The optimized catechin compounds were docked to HER-2 protein through Autodock Tools using the same procedures and coordinates as when being validated. Catechin compound docking on Her-2 protein obtained ten poses. Then, the lowest binding energy value, showing the most stable binding, among the poses was selected. The docking result reveals that the binding energy

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between catechins and HER-2 protein is negative. However, the catechins had higher binding energy than the native ligand. This indicates that the potential of catechins in binding the active sites of HER-2 is weaker than the native ligand, but catechins still have the ability to bind HER-2 receptors.

Table 1: Docking results between Catechins with HER-2 receptor

Ligand	ΔG (kcal/mol)
Epigallocatechin-3-gallate (EGCG)	-6.94
Epicatechin-3-gallate (ECG)	-7.07
Epigallocatechin (EGC)	-7.02
Epicatechin (EC)	-7.63
Native ligand	-10.48

The value of binding free energy is shown in Table 1. The important residues in the native ligand, SER728, MET801, THR862 and ASP863, can be seen in Table 2. Based on the docking result, catechin compounds can interact with HER-2 protein through hydrogen bond in SER728, MET801, THR862 and ASP863 amino acids (shown by the green circle in Fig. 2). This indicates that the active sites between the native ligand and the catechin in HER-2 protein are the same so that they will produce the same activities as the native ligand in inhibiting HER-2 protein. Visualization of the docking result and the interaction between catechin with HER-2 protein are shown in Fig. 2 and Table 2.

From Ashtekar's research, it was found that ASP863 is a very important amino acid residue in HER-2. Of the several HER-2 inhibitors, only Lapatinib and Neratinib interact with ASP863 residue, which is why both drugs specifically target HER-2(17). In this study, information was obtained that three catechin compounds namely EGCG, EGC, and ECG can interact with ASP863 residue

Table 2 : Atomic Interaction Between Ligands with The Kinase domain of HER-2 receptor

Ligand	Atomic Association		Amino Acid Residue	Distance (Å)
	Ligand Atom	Receptor Atom		
Epigallocatechin-3-gallate (EGCG)	O	NH1	ARG811	2.81188
	H	OD2	ASP808	1.89325
	H	OD1	ASN850	1.74509
	H	O	ARG849	2.07933
	H	**OD2**	**ASP863**	**2.27632**
	H	**OD2**	**ASP863**	**2.10809**
Epicatechin-3-gallate (ECG)	H	OD2	ASP808	2.24254
	H	OD1	ASP808	2.48394
	H	**OD2**	**ASP863**	**2.19663**
	H	O	ARG849	2.17508
	O	N	CYS805	2.57212
	H	**O**	**MET801**	**2.06650**
	O	**N**	**MET801**	**2.66332**
Epigallocatechin (EGC)	O	N	CYS805	2.74111
	H	OD1	ASP808	2.15689
	H	OD1	ASP808	1.77005
	H	O	LEU726	2.35829
	O	**OG1**	**THR862**	**3.05302**
	H	**OD2**	**ASP863**	**1.76747**
Epicatechin (EC)	**H**	**O**	**SER728**	**1.93489**
	H	**O**	**SER728**	**2.22756**
	O	**N**	**SER728**	**2.95638**
	H	OD1	ASP808	1.86508
	O	N	CYS805	2.87535
	O	**N**	**MET801**	**2.81018**
	H	**O**	**MET801**	**1.9474**
Native ligand	N	N	MET801	2.86028
	O	O	SER728	2.89546
	N	OG1	THR862	2.92737
	N	N	ASP863	3.06601

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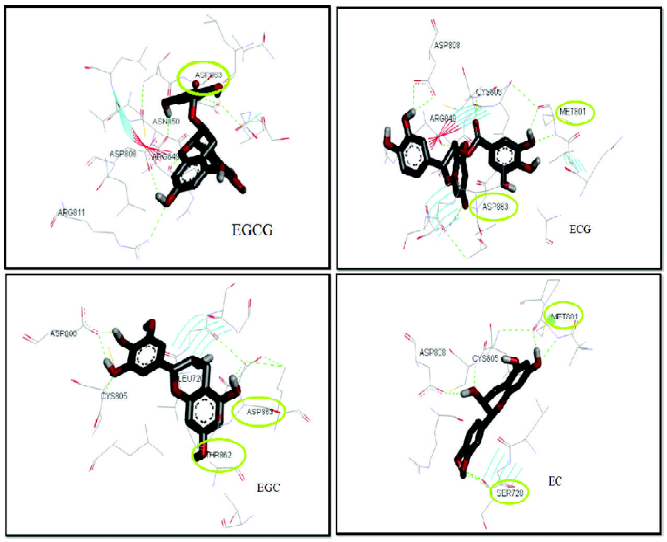

Fig. 2. Catechin interactions with the kinase domain of HER-2

thus three compounds are potential hits to develop as HER-2 inhibitors.

Molecular dynamics

Molecular dynamic simulation is crucial for understanding changes of protein conformation over time (18). Molecular dynamics was conducted to evaluate the dynamic behaviour of catechins in the complex with kinase domain of HER-2 (PDB ID: 3PP0) during 10 ns. This study analyses some parameters such as RMSD protein-ligand, RMSF and the formed protein-ligand interaction.

RMSD analysis is used to obtain insight of the structural conformation happened to protein during simulation. The plot for HER-2 protein versus time (10 ns) for the simulation is shown in Fig. 3. Based on the Fig. 3, it can be seen that the native ligand complex has lower RMSD value than the catechin-receptor complex. This indicates that the structure of the native ligand complex is more stable.

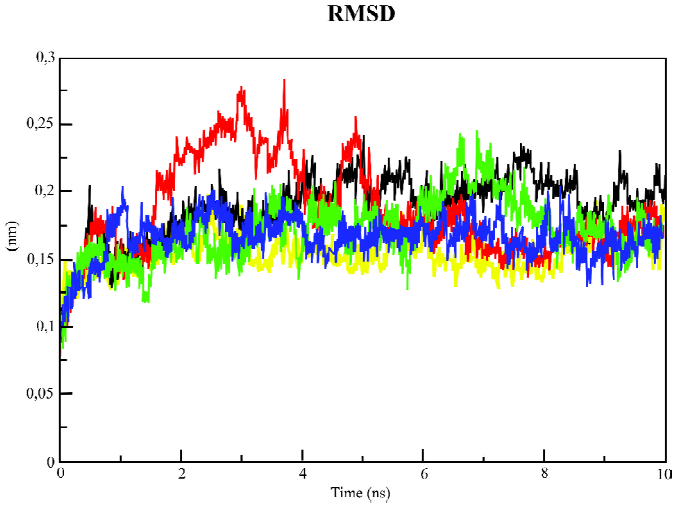

Fig. 3. RMSD plot of each ligand-HER2 complex during 10 ns simulations. EC (black), ECG (red), EGC (green), EGCG (blue), and native ligand (yellow).

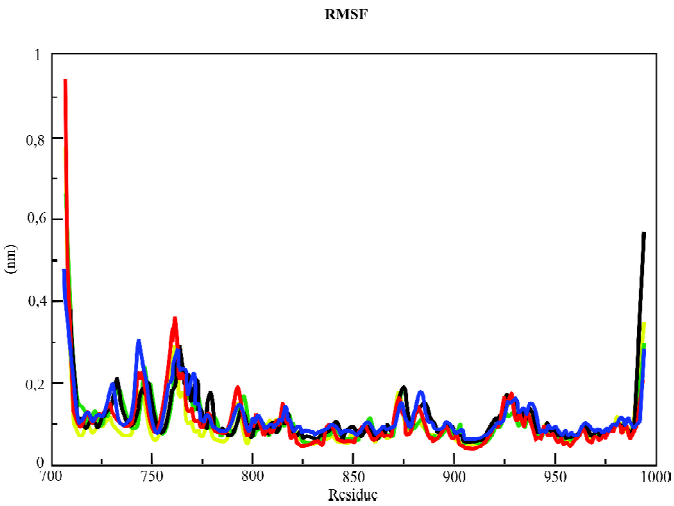

Fig. 4. RMSF plot of each amino acid residue during 10 ns simulation. EC (black), ECG (red), EGC (green), EGCG (blue), and native ligand (yellow).

RMSF is calculated for every amino acid residue of protein, which is to see the extent of fluctuations in the movement of each amino acid residue during the simulation. The plot for ligand RMSF (nm) versus ligand atom index is shown in Fig. 4. The low flexibility of amino acid residues shows the stable interaction in the active site bond to the test compound. This is because the atoms forming the amino acid residues tend not to change much from their position during molecular dynamic simulation. From Fig. 3, it can be seen that native ligand complex (native ligand-HER-2) has the lowest value compared to catechin complex (Catechin-HER-2). This indicates that native ligand?HER-2 complex has a better stability than catechins complex.

To assess the affinity of each catechin compound against HER-2, the calculation of binding free energy was conducted using MM-PBSA method (19). Binding free energy is the total result of several components, namely electrostatic, van der Waals, and non-polar desolvation energy, in the interaction of receptor-ligand which can be calculated directly using some conformations of the simulation result trajectory. Table 3 shows that bond free energy of receptor HER-2- native ligand complex is lower than HER-2catechin complex. This indicates that catechin compounds have a bad affinity against HER-2 receptor.

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Table 3 : Binding Free Energy

Ligand	Binding free energy (ΔG) (kJ/mol)	
	Molecular Docking	**Molecular Dynamic**
Epigallocatechin-3-gallate (EGCG)	-29.03696	-64.709 +/- 11.880
Epicatechin-3-gallate (ECG)	-29.58088	-87.123 +/- 16.580
Epigallocatechin (EGC)	-29.37168	-50.341 +/- 11.212
Epicatechin (EC)	-31.92392	-58.757 +/- 14.551
Native ligand	-43.84832	-153.205 +/- 12.199

There are differences in the value of binding free energy obtained from the docking and molecular dynamic results, see Table 3. Based on the energy value, epicatechin-3-gallate (ECG) has the best binding energy among the other catechin compounds. This result is in line with the number of the interaction between ECG and HER-2 receptor. Even though the catechin compounds have a lower affinity than the native ligand, they have the same active site which is characterized by the similarity of several amino acid residues that interact with HER-2. In addition, three catechin compounds can also interact with ASP863 which is an important residue in HER-2 (17). Therefore, catechin compounds, especially EGCG, EGC, and ECG, are potential to be developed into candidates for breast cancer drugs as HER-2 inhibitors through structural modification to increase interaction with HER-2 receptor.

4. Conclusion

In this study have done molecular docking and molecular dynamics simulation. The molecular docking and molecular dynamic results show that the catechins can interact with the HER-2 receptor, but the binding free energy of the catechin-HER-2 receptor complex is higher than the native ligand-HER-2 receptor complexs. This indicates that the catechin compounds has a poor affinity for HER-2 receptors.The Catechin compounds binding several amino acid residues that are the same as in native ligands, this indicates that they are binding to the same active site. In addition, three catechin compounds can also interact with ASP863 which is an important residue in HER-2 (17). Therefore, catechin compounds, especially EGCG, EGC, and ECG, are potential to be developed into candidates for breast cancer drugs as HER-2 inhibitors through structural modification to obtain catechin derivative compounds.

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Conflict of Interest

The author declaire that there is no conflict of interest.

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Revisiting the Intractable Barriers Affecting Medication Adherence Among Outpatients with Schizophrenia

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Abstract

Medication adherence is one of the foremost problems affecting antipsychotic efficacy in schizophrenia patients. Medication nonadherence among schizophrenia patients has been often estimated > 50%, leading to higher rates of relapse and hospitalization as well as to decreasing cognitive and functional prognosis. The purpose of the study is to identify the strategy for improving medication adherence in schizophrenia and evaluate adherence using Medication Adherence Rating Scale (MARS) and determinant factors affecting adherence. Prospective study with cross sectional design was conducted from October to December 2019. Especially data from schizophrenia outpatients in one of national mental hospital in Indonesia. Schizophrenia outpatients were majority male (60%), the age range from 31-49 years were 70%, most of patients are single (63,33%), 70% have secondary education, 70% of them are from Surabaya area, and half of them their duration of the disease from 1 to 5 years. This study showed that the pattern of prescription of antipsychotics are risperidone and clozapine were the most antipsychotics prescribed. 40% of patients have good adherence, 40% of patients have partial adherence, and only 20% of patients' poor adherence. Most of schizophrenia outpatients have experience in forget to take his/her medicine and careless at times about taking his/her medicine and less knowledge about schizophrenia. In other hand, 100% patients have agreed by staying on medication, it can prevent getting sick. The mental hospital should utilize educational program to improve patient's awareness about their disorder and their medications to improve their adherence.

Key words : Schizophrenia,Antipsychotics, SGAs, Medication Adherence, MARS, Mental Health

1. Introduction

It is not easy to maintain medication adherence on patient with schizophrenia. Although the advances in psychopharmacology have greatly improved the range of options for treating schizophrenia, the outcome may not be optimal to prevent patient from relapse and hospitalization (1). Low adherence has been evident in many patients with schizophrenia contributing to a number of severities including higher risk of suicide and financial burden which affects not only patients but also their families and care givers (2). Several publications have reported significant portion of non-adherence in the case of schizophrenia ranging from 40% to 70% (3). In fact, 75% of patient with schizophrenia stop taking their medication within 18 months (4).

Poor adherence in schizophrenia can be associated with a number of factors such as social isolation, stigmatization and comorbidities substance misuse of psychotropic medication (5). As adherence is a complex phenomenon, these factors may be exacerbated by a wide variety of other causes such as lack of illness awareness, the adverse effect of the medication, the long-term treatment and the fragmented health care services for patient with mental health issues. Such condition may be increased yet undetected in the outpatient setting as patient will need to undertake and be responsible for the medication at their own risk (5).

The risk of non-adherence in the outpatient setting cannot be neglected. A systematic review of longitudinal studies reported that there were 27% of individuals with schizophrenia who had poor outcome after the first episode of psychosis (6). Another study indicated that 82% of patients would likely to suffer first relapse and 78% would continue to suffer the second relapse after the first episode of psychosis (7).

The causes of non-adherence include the patients factors as fear of adverse effects, physical and psychiatric conditions, forgetfulness, external distractions, misunderstanding instructions, lack of insight and lack of information about disorders. Treatment factors as numerous medications, enduring symptoms, partial or no efficacy. Social economic factors as lack of income, transportation, living alone, and stigma of mental illness (8).

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Schizophrenia is one of nine chronic disease covered by national health covered in Indonesia (9). In Indonesia, the number of relapse on schizophrenia patients was reported. The number of relapsed had significant correlation with medication non-adherence. The common problems of medication non-adherence among schizophrenia patients in Indonesia were social economic, attitudes to medication, knowledge, and family support (10).

Medication non-adherence will escalate the risk of recurrences, hospital admission rate and medication expense (11). The cost of re-hospitalizations and non adherence per year were 100 billion USD and 290 USD (12). In Indonesia, cost of illness schizophrenia was estimated 32 million IDR/year/patient (13). The objective of this study is to identify the strategy for improving medication adherence in schizophrenia and evaluate adherence using Medication Adherence Rating Scale (MARS) and determinant factors affecting adherence.

2. Materials and Methods

Design **:** Prospective study with cross sectional design was conducted from October to December 2019. This study has been approved by the Ethic Committee of the Menur Mental Hospital with number of ethical approval 070/7556/305/2019. Especially data from schizophrenia outpatients in one of national mental hospital in Indonesia. Non probability sampling (purposive sampling) all schizophrenia patients who registered as an outpatient national mental hospital in the chosen sitting and fulfill the inclusion criteria was selected.

Subjects **:** The inclusion criteria are patient with schizophrenia, being adult aged 18 or older, who agree to participate in the study, and patient who have insight. The exclusion criteria are patient who have other mental disorder and patients diagnosed with brain dysfunction or cognitive impairment. The minimum sample size for descriptive quantitative research not less than 30. The participants were 30 patients. Informed consent was obtained from all participants after explaining the study and its objectives. Participants were included only after they signed the informed consent. All researchers ensured participant data confidentiality and compliance with the Declaration of Helsinki. This study was conducted in one of national mental hospital in Indonesia. Participants were interviewed regarding their history of mental illness, sociodemographic characteristic, and pharmacological treatment.

Instrument **:** The Medication Adherence Rating Scale (MARS) tool was used for measuring level of adherence. It was formerly evolved and validated by Thompson et al., to evaluate treatment compliance specifically in people under antipsychotic treatment. It was designed to assess both the patients attitude towards medication and also actual medication taking behavior. the reliability analysis of the MARS using cronbach's alpha was 0.75 (14). The validity and reliability of MARS with large sample (N=319) by Fond et al., a coefficient were close to 0.6 (15). It was translate into Indonesia and validated by Yuliana et al., with reliability result 1.107 (16).

MARS consists of three parts questions/statements; question 1-4 represent treatment adherence behavior, question 5-8 represent attitude toward taking medicines and question 9-10 represent adverse effects and attitudes to antipsychotic treatment. Every question or statement should be answered with a 'YES' or 'NO' answer. A negative response indicate with non-compliance is code as zero. Whereas a positive response indicate with compliance is coded as one.

For questions 1-6 and 9-10 an 'disagree' answer is indicate of positive response and hence should be coded as one. In opposed for questions 7-8 a 'agree' answer pointing to positive response and hence should be coded as one. The whole of adherence scoring range between nil (non-compliance) to ten (compliance), with a greater score pointing good attitudes and behavior towards positive compliance. Patient with total score < 5 (non adherence), 5-7 (partial adherence), and ? 8 (good adherence).

Processing and analyzing data **:** Statistical Package for social Science (SPSS) version 24 was used. The following statistical measured were used as descriptive measures as numbers, percentage, mean and standard deviation. Analytical statistics as T-test independent sample and Analysis of Variance one away.

3. Results and Discussion

Characteristic of schizophrenia outpatients and medication

Table 1 reveals that male were majority (60%), the age range from 31-49 years were 70%, most of patients are single (63,33%), 70% have secondary education, 70% of them are from Surabaya area, and half of schizophrenia patients have mental disorder with a range duration of 1 to 5 years. Table 2 shows that the pattern of prescription of antipsychotics are risperidone and clozapine were the most antipsychotics prescribed for schizophrenia

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Table 1. Characteristic of schizophrenia outpatients

Characteristics	N	%
Gender		
Male	18	60
Female	12	40
Age (year)		
18-30	4	13.3
31-49	21	70
50-65	4	13.33
>65	1	3.33
Marital status		
Single	19	63.33
Married	10	33.33
Divorced	1	3.33
Educational level		
Elementary school	4	13.33
Junior high school	9	30
Senior high school	12	40
College or higher	5	16.67
Occupation		
Full time	11	36.66
Part time	5	16.67
Not worker	14	46.67
Duration of treatment (year)		
1-5	15	50
6-10	10	33.33
11-15	5	16.67
Number of antipsychotic		
Monotherapy	4	13.33
2 antipsychotics	23	76.67
≥ 3 antipsychotics	3	10
MARS total score		
Minimum total score	4	
Maximum total score	10	
Mean total score	7.20	

Table 2. Regimen of oral antipsychotics

Regimen therapy	N	%
Risperidone	3	10
Risperidone + Clozapine	12	40
Risperidone + Clozapine + Trifluoperazine	3	10
Clozapine + Trifluoperazine	8	26.67
Haloperidol	1	3.33
Haloperidol + Clozapine	2	6.67
Aripiprazole + Clozapine	1	3.33

outpatients. Currently, atypical antipsychotics became drug of choice in schizophrenia treatment considering more effective in relapse prevention, reduced risks of extrapyramidal syndrome and increased quality of life (17).

Level of adherence and attitude towards medication

The effectiveness of the medication is impacted and the chance of recurrence will be elevated when people with schizophrenia discontinue taking medication. The results of this study, only six patients (20%) have poor adherence (see figure 1). This results was in the line with Kamali et al., who reported that schizophrenic subjects had poor adherence lower than schizophrenic subjects had good adherence (18). Contrasting to other studies, who informed that lack of compliance was found in about half of people suffering from schizophrenia (19,20,21).

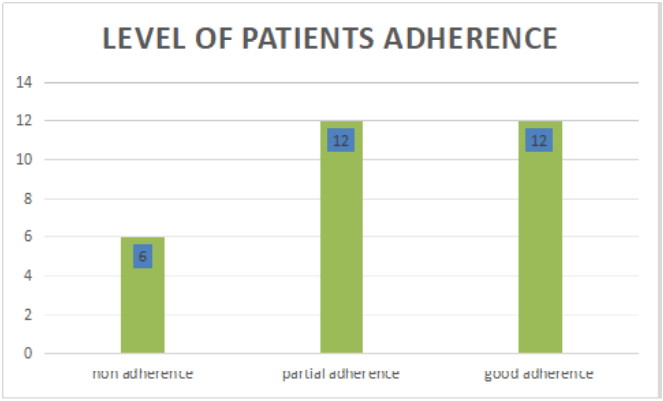

Figure 1. Number of level of patients adherence

In other hand, table 3 indicated that most of patients with schizophrenia undergo in forget to take his/her medicine and careless at times about taking his/her medicine, adverse effects and deficiency of insight and shortage of information about their disorder. From the sighting, this is might because the healthcare team did not provide the client and caregiver the comprehensive information according to their treatment and illness. Including intervention, dosage regiment, therapeutic effect, and adverse effect. The results of current study indicated that half participants with schizophrenia disorder did not clearly understand their illness and medication.

Many studies has reported that medication adherence is related to knowledge and experience of, and insight into the illness, in addition to patient's attitudes toward the use of medication for the treatment of psychiatric disorders (22,23,24,25). One of study reported that when patients were not fully informed about their illness and treatments, there were likely to discontinue medication therapy of their own volition without discussing the matter with healthcare professional (23).

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Table 3. Frequency of Attitude towards medications

Medication adherence questions/statement	YES		NO	
	N	%	N	%
1. Do you ever forget to take your medication?	15	50	15	50
2. Are you careless at times about taking your medicine?	13	43.3	17	56.7
3. When you feel better, do you sometimes stop taking your medicine?	6	20	24	80
4. Sometimes, if you feel worse when you take the medicine, do you stop taking it?	4	13.3	26	86.7
5. I take my medicine only when I am sick	2	6.7	28	93.3
6. It is unnatural for my mind and body to be controlled by medication	3	10	27	90
7. My thoughts are clearer on medication	16	53.3	14	46.7
8. By staying on medication, I can prevent getting sick	30	100	0	0
9. I feel weird, like a 'zombie', on medication	9	30	21	70
10. Medication makes me feel tired and sluggish	19	63.3	11	36.7

Factor affecting medication non-adherence

The triggers of non adherence include the personal factors, medication factors, and socio-economic environment factors. The results of this study there is no significant different adherence score between gender group and educational level group (table 4). This finding inline with Naafi et al., who reported there is no meaningful difference between patients characteristic and the patients medication adherence level (26). Conforming current study pointed out that there is no meaningful different between treatment factors such as duration of treatment and number of antipsychotics with treatment compliance score (table 5).

Table 4. Independent sample T-test gender different

Gender	N	adherence score (mean)	SD	Sig.(2 tailed)
Male	18	7.33	1.847	.564
Female	12	7.00	1.279	
Total	30	7.20	1.627	

This study contrasting to Dibonaventura et al., Dassa et al., and Yang et al., who reported that the quantity of medications may affects patient's toward compliance (16,17,18). The results of this current study shows more than 50% of participants has experiences with antipsychotic side effects. Lack information or education about heir medication and side effect might be occur during treatment has negative impact on their treatment compliance. One of study reported that pharmacist counseling there was meaningful difference adherence level between pre and post pharmacist counseling intervention (27). Therefore, the health care team should give the patient and/or the caregiver psycho-educational program for compliance of treatment improvement (28,29,30).

Table 5. Analysis of Variance One Away

	Adherence			
Studied variable	N	Adherence score (mean)	SD	Sig
Education lavel				
Elementary school	4	7.50	2.380	.942
Junior high school	9	7.00	1.732	
Senior high school	12	7.33	1.303	
College or higher	5	7.00	2.000	
Total	30	7.20	1.627	
Treatment duration				
1-5 year	13	6.92	1.847	.307
6-10 year	12	7.75	1.357	
11-15 year	5	6.60	1.517	
Total	30	7.20	1.627	
Number of antipsychotic				
1 antipsychotic	4	7.75	1.500	.307
2 antipsychotics	23	6.96	1.692	
≥ 3 antipsychotics	3	8.33	.577	
Total	30	7.20	1.627	

There are several limitations in this study. Due to limited sample size and lack of clinical data as adherence parameter. Prospectively study with various number sample size and objective parameter of adherence might be considered. Despite the several limitations, our study provides preliminary finding to explore barriers affecting medication adherence in mental health disorder treatment.

4. Conclusion

Adherence to medication is a critical issue for patients with mental disorder. It cannot be overemphasized that patients should have insights into their own mental disorder and realize the necessity of taking medications to improve their chances of a successful recovery. The

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healthcare provider should empower counseling program to elevate patient's cognition about their illness and their treatments affecting their compliance. Future research should focus on pharmaceutical care intervention such therapeutic monitoring and education of schizophrenia disorder and medication to improve patients knowledge of disorder and medication and attitudes toward medications.

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Conflict of interest

The authors declare no financial or commercial conflict of interest.

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A Production and Activity Test of Anti-bacterial Compounds of Endophytic Fungi BR-S1 (a) Isolate Extract in Different General Growth Media

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Abstract

BR-S1 (A) isolate is an endophytic fungi isolated from the medicinal plant of tea parasite (Scurrulaoortiana) which is estimated to contain anti-bacterial compounds. The research questions are as follow; can an anti-bacterial compound be produced in general growth medium, and is it effective in inhibiting or killing MRSA bacteria pathogen.The aim of this research is to find out the types of general growth media that can be used to produce anti-bacterial compounds and to determine the effectiveness of these compounds in inhibiting or killing pathogenic MRSA bacteria.This research was conducted using laboratory experimental methods with the main variables in forms of three different types of general growth media, namely the Potato Dextrose Broth (PDB) medium, the CzapekDox Liquid Medium (CDLM), and the Malt Extract Broth (MEB) medium. The results show that the three types of general growth media were not able to stimulate the production of anti-bacterial compounds which were characterized by the absence of discoloration and medium turbidity and the absence of thick mycelium growth. The results of anti-bacterial activity tests on pathogenic MRSA bacteria show no inhibition zone formed around the disc paper added with endophytic fungi extracts, whereas positive controls formed inhibitory/clear zones.The production process of BR-S1 (A) isolate anti-bacterial compound was influenced by three factors, namely the composition and chemical properties of the medium, age and number (concentration) of cells, and environmental conditions (temperature and aeration) of the production site. It can be concluded that the three types of general growth media of PDB, CDLM, and MEB cannot be used as a medium for the production of anti-bacterial compounds. The antibacterial compounds produced by BR-S1 (A) isolates are not effective in inhibiting pathogenic MRSA bacteria.

Key words : Anti-bacterial compounds · endophytic fungi · and general growth medium

1. Introduction

Indonesia is one of the countries with the greatest biodiversity in the world which owns various types of plants, animals, fungi, and bacteria with uncover its potential. One real step to do is through the exploration and management of various types of plants as a source of medicine or medicinal raw materials.

Tea plant parasite is one of the plants being explored and collected recently as a source of medicine or medicinal raw materials. Based on several results of previous studies, it is believed that the ability of the parasite plant in treating various diseases is related to the contained active compounds.

Although the potential of the tea plant parasite is covered, direct utilization of this plant as a source of medicine or medicinal raw materials is apparently not easy and is constrained by several factors such as the its limited number, requires large biomass, and the nature or structure of the active compound which is very sophisticated. To overcome these obstacles, endophytic fungi which lives within these plants is utilized as a source of medicine or medicinal raw materials.

Endophytic fungi or molds are molds which have been whole or part of their life cycle by colonizing healthy tissue from host plants both intercellularly and intracellularly without causing disease with obvious symptoms (2). As the most dominant group, endophytic fungi have great potential to be developed related to its ability of several types of molds which are able to produce several active compounds such as antibiotics, antiimmunosuppressive, antidiabetic, anticancer, antiinsecticidal, and antiviral in a wide range.

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The results of (5) discovered that in the tea plant parasite, 17 endophytic mold were successfully isolated. The results of testing crude extracts from 17 isolates showed that there were only 5 isolates which presented positive results in inhibiting bacterial growth. The ability of endophytic molds to produce secondary metabolites (antibacterial compounds) on a laboratory scale (in vitro) is affected by two main factors; physical factors such as pH, temperature and incubation time and chemical factors such as nutrient content available in growth media such as sources of N, C, amino acids, and other additional nutrients.

Study on the correlation or effect between types of fermentation media with the ability to produce antibacterial compounds from endophytic fungi isolates needs to be done. It is essential because this study will assist to find the most appropriate or optimal medium for the production of antibacterial compounds. Later, the results of this production can be tested onMRSA bacteria, a special strain of the bacterium Staphylococcus aureus which has resistance to beta-lactam antibiotic group which includes methicillin, oxacillin, penicillin, amoxicillin, cephalosporin, and carbapenem.

Based on the description above, a number of problems can be formulated as follows :

1. What types of fermentation medium can be used by endophytic fungi isolates BR-S1 (A) in producing antibacterial compounds?
2. Which type of fermentation medium is the most optimal in producing antibacterial compounds from endophytic fungi isolates BR-S1 (A)?
3. How effective are antibacterial compounds produced by endophytic fungi isolates BR-S1 (A) in inhibiting the growth of MRSA bacteria?

This study is expected to provide information about the types of medium which can be used and the most optimal in producing antibacterial compounds and discover its effectiveness in inhibiting the growth of MRSA bacteria.

The objectives are as follow:

1. To discover the types of fermentation medium which can be used by endophytic fungi isolates BR-S1 (A) in the production of antibacterial compounds
2. To determine the most optimal type of fermentation medium in producing antibacterial compounds from endophytic fungi isolates BR-S1 (A)
3. To present the effectiveness of antibacterial compounds produced by endophytic fungi isolates BR-S1 (A) in inhibiting the growth of MRSA bacteria

This research is expected to provide information about the types of medium that can be used and the most optimal in producing antibacterial compounds and to illustrate how much effectiveness in inhibiting the growth of MRSA bacteria.

2. Materials and Methods

Material and research objectives **:** The toolsutilized in this study are markers with 0.5 cm and 1.8 cm diameter holes, petri dishes, incubators (Memmert INB 400), Laminar Air Flow (LAF), test tubes, Erlenmeyer flask, rotary shakers (Kottermann 4010), thermometer, separating funnel, vacuum pump, tapered erlenmeyer, measuring cup, stirrer hotplate (Barnstead SPI 31320-33), oven (Memmert UNB 400), analytical balance (AND GR-200), micropipettes (Boeco), refrigerator (LG expresscool), water bath, calipers, autoclaves (All America 25X) and centrifuges.

The materials used in this study included samples of the tea plant parasite, tap water, 75% ethanol, 5.25% sodium hypochlorite (bayclin), sterile distilled water, filter paper, cotton, 1% streptomycin antibiotics, Potato Dextrose Agar (PDA) medium (Oxoid), Manitol Salt Agar (MSA) medium (Oxoid), Plate Count Agar (PCA) medium (Oxoid), Potato Dextrose Broth (PDB) medium (Oxoid), Tryptic Soya Agar (TSA) medium (Merck), Tryptic Soya Broth (TSB) medium (Merck), CzapekDox Agar (CDA) medium (Oxoid), Malt Extract Agar (MEA) medium (Oxoid), CzapekDox Broth (CDB) medium (Oxoid), Malt Extract Broth (MEB) medium (Oxoid), Mueller Hinton Agar (MHA) medium (Merck), paper disk blank (Oxoid), ethanol 96%, antibiotic vancomycin, fungi isolates endophytic BR-S1 (A) and MRSA bacteria.

Research design and data analysis **:** This research was conducted by laboratory experimental methods with independent variables in the form of different carbon sources (C) contained in growth media such as dextrose (PDA/PDB), sucrose (CDA/CDB) and Malt extract (MEA/MEB). The dependent variable in this study was the ability of BR-S1 (A) endophytic fungi isolates to produce antibacterial compounds.

Endophytic fungi cultivation in tea plant parasite (8) with modification **:** Isolate endophytic fungi BR-S1 (A) were grown on PDA medium and incubated for 7-14 days at room temperature assuming on the 14th day the entire

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surface of the medium was overgrown and covered by mycelium. Then, the PDA medium was sampled using a hole diameter of 0.5 cm to be inoculated on the PDA, MEA, and CDA medium with each medium planted with 1 sample right in the middle of the petri dish and with the reverse position of the sample so that the mycelium directly touched the surface of the medium. All subsequent mediums were incubated at room temperature with the petri dish turned upside down for 7-14 days and each medium was repeated 3 times.

Characterization and measurement of growth of tea parasite molds (3) **:** The morphological character of BR-S1 (A) endophytic fungi isolate in all types of cup medium was observed both macroscopically and microscopically and their growth rate was measured every day. Macroscopic observations were the color and texture of the surface of the colony, the color behind the colony, the edge of the colony, the shape of the colony, the presence or absence of zoning, the growing area, radial lines, exudate drops and its color, and the organs formed (fruiting body, schlerotia, synnema, sporodochia, stroma, and setae). Microscopic observations were the presence sectional hyphae, hyphae pigmentation, presence or absence of clam connections, forms of hyphae modification (spiral, nodular organ, pectinate body, antler hypha, racquet hypha), the presence of rhizoid , asexual spores (simple shape, special shape, size, arrangement), and the presence of sexual spores like ascospores, basidiospores, andzigospores).

MRSA test bacteria reaction (6) with modification **:** Reaction of the bacterial inoculum test was carried out by the Standard Plate Count (SPC) method. 1 ml stock of MRSA bacteria was taken to be added to 25 ml of a sterile TSB medium and shaker at 100 rpm for 1x24 hours at 37 oC. From the TSB medium, the dilution was carried out up to 10-7 dilution level with the last two dilutions being duplicated plating by Pour Plate method on the MHA medium. Then, the MHA medium was incubated for 1x24 hours at 37 oC and counted the number of colonies growing on each petri dish.

Extraction and antibacterial activity test of tea plant parasite (8) with modification **:** All liquid media were removed from the rotary shaker and the results of cultivation were filtered by filter paper (Whatman No. 1) so that mycelium and filtrate would be obtained later. The pH of the filtrate was measured by pH meter and then centrifuged at a speed of 3,000 rpm for 20 minutes to obtain natant and supernatant. Supernatant was collected and put into microtube for centrifugation at a rate of 13,000 rpm for 15 minutes. The obtained supernatant was then used to test antibacterial activity. As a negative control, a sterile liquid medium was utilized by extracting the same method as above.

Antibacterial activity test of crude extract of endophytic fungi isolate BR-S1 (A) was carried out through agar diffusion method. It was prepared a cup of MHA medium which had previously been inoculated with 1 ml of overnight MRSA bacteria and allowed to solidify. On top of the medium, it was then placed 7 pieces of 6 mm in diameter sterile disk paper, each dropped with 20 µl filtrate/extract of endophytic mycelium fungi (6 disc paper) and vancomycin antibiotics (1 disc paper) as positive control. The medium MHA cup was then incubated at 37°C for 1-2 x 24 hours and observed whether there were any inhibitory zones (clear zones) formed around the disk paper. Inhibition zones formed were measured by a calipers diameter and so did the diameter of the paper discs used. Based on the diameter data obtained, the areas of inhibition zone were calculated by the following formula :

$$L_{zhaw} : \pi . r_a^2$$

$$L_{zhak} : L_{zhaw} - L_{kc}$$

$$L_{kc} : \pi . r_b^2$$

Information :

r_a : Radius of inhibition zone (mm)
r_b : Radius of paper disk (mm)
L_{zhaw} : Area of initial inhibition zone (mm^2)
L_{kc} : Area of disc paper (mm_2)
L_{zhak} : Area of the final inhibitory zone (mm^2)
Π : 3,14

3. Results and Discussion

Cultivation of endophytic fungi in tea plant parasite: In this study, three types of fermentation media were PDA or PDB medium, MEA or MEB medium, and CDA or CDB medium. From the three types of mediums, it could be seen that the endophytic fungi of the tea plant parasite isolate BR-S1 (A) had the fastest growth in the PDA or PDB medium, followed by the CDA or CDB medium and finally the MEA or MEB medium (Figure 1)

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Extraction and antibacterial activity test for endophytic fungi of tea plant parasite Figure 1 Figure 2

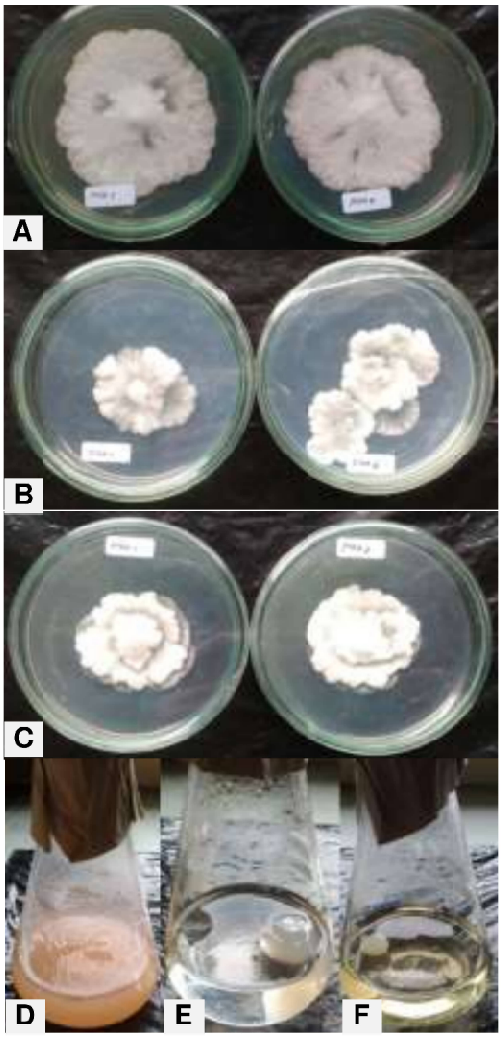

Figure 1. Growth rate of endophytic fungi isolates BR-S1 (A) in several types of fermentation medium. A). PDA medium; B). CDA medium; C) MEA medium; D). PDB medium; E). CDB medium; F). MEB medium.

Table 1. Inhibition zones calculation of antibacterial activity test for endophytic fungi BR-S1 (A) extract against MRSA bacteria from three different types of liquid fermentation medium.

Disk No	Repetitions (mm^2)				
	U_1	U_2	U_3	Σ	average
1	0.00	0.00	0.00	0.00	0.00
2	0.00	0.00	0.00	0.00	0.00
3	0.00	0.00	0.00	0.00	0.00
4	0.00	0.00	0.00	0.00	0.00
5	0.00	0.00	0.00	0.00	0.00
6	0.00	0.00	0.00	0.00	0.00
7	190.67	205.33	252.15	648.15	216.05

Information table 1:

1. Negative control of PDB extract
2. Negative control of CDB extract
3. Negative control of MEA extract
4. MEB Extract
5. CDB Extract
6. GDP Extract
7. Positive vancomycin control

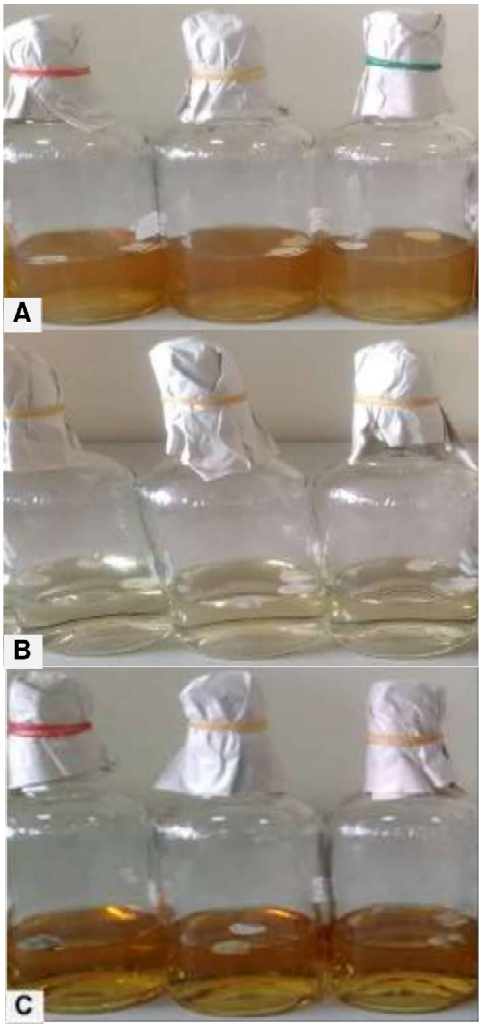

Figure 2. Results of endophytic fungi fermentation of BR-S1 (A) isolates in three different types of fermentation media. A). PDB medium; B). CDB medium; and C). MEB medium.

The fermentation process of endophytic fungi isolate BR-S1 (A) was carried out on PDB, CDB, and MEB mediums with an incubation time of 7 days at 30 oC in a 100 rpm incubator shaker. This research selected liquid fermentation medium considering the easy regulated and determined liquid medium, its homogenecity and can provide optimal conditions for cell growth of its composition and concentration, and the use of the medium becomes more efficient.

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From figure 1 we know that these three types of medium were the same common medium in fact and used for the cultivation of various types of mold or fungi. However, its energy sources (carbon) had a different composition. The PDA or PDB medium was composed from potato extract and dextrose (glucose), the CDA or CDB medium was composed from sucrose as the only source of carbon and the MEA or MEB medium was composed from malt extract containing polysaccharides.

PDA or PDB medium was the medium which obtained the simplest carbon source, namely the monosaccharide group in the form of dextrose (glucose) which could be directly absorbed and utilized by endophytic fungi to stimulate cell growth. It was different from CDA or CDB and MEA or MEB medium which collected carbon sources in the form of disaccharides (sucrose) and polysaccharides (maltose) which could be utilized by endophytic fungi to be broken down first into simpler monosaccharides. Previous research suggested that mold species could utilize some of the carbon sources contained in the medium for vegetative cell growth and the availability of simple carbon sources which would be relatively easily digested and could increase their metabolic processes (11).

The fermentation results of endophytic fungiisolates BR-S1 (A) could be seen in figures 2 above. From the picture, it could be understood that the fermentation process did not occur optimally, this could be seen from the color of the fermentation medium which did not turn out to be thicker or darker compared to the sterile fermentation medium. In addition, samples of endophytic fungi mycelium planted in the fermentation medium did not grow and multiply.

The fermentation process of endophytic fungi was affected by several factors such as the composition and chemical properties of the medium, age and number (concentration) of cells added to the fermentation medium, and the environmental conditions in which fermentation occurred. From these factors, age and number (concentration) of cells and environmental conditions which contributed the fermentation process in this study did not optimally occur.

Considering from the number (concentration) of cells used, this study was more than enough because every 1 bottle of 150 ml volume fermentation medium had been filled with 3 samples of endophytic fungi mycelium, each 1 cm in diameter. On the contrary, considering from the age of the cells, endophytic molds utilized were old (more than 4 weeks) and hadcome the phase of death (decline). In this phase, metabolic activity had decreased so that the nutrient content contained in the medium could not be converted and utilized by endophytic molds to stimulate cell growth.

Another factor which also affected the fermentation process was bad condition of the fermentation environment. During the fermentation process, the temperature inside the incubator shaker had been set at room temperature (28^{o}C), but in reality the temperature inside the incubator shaker reached hotter (higher) than room temperature (28^{o}C). Previous research suggested that temperature was one of the environmental factors which could affect the life and growth of mold cells (11). Fungi growth was affected by several factors and one of them was environmental temperature. Normally, mold could grow optimally in the temperature range of 25^{o}C - 35^{o}C. A higher temperature than the optimal temperature could cause mold cells to damage and die (1).

The extraction process of fermented endophytic molds of BR-S1 (A) isolates was carried out through filtration of vacuum pump and filter paper. In this process, it was obtained in the form of supernatant fluid without the filtrate of endophytic fungi mycelium. The results of this extraction process were further processed to test the antibacterial activity against MRSA bacteria.

Figure 3 and table 1 above described the extract of endophytic fungi isolate BR-S1 (A) from fermentation on three different types of liquid fermentation media had no ability to inhibit/kill MRSA bacteria characterized by the absence of inhibitory zones formed around disc paper. Inhibition zones were only formed on paper disk No. 7 administered with vancomycin antibiotics as a positive control.

The inability of endophytic fungiextract of BR-S1 (A) isolates to inhibit/kill MRSA bacteria might be affected by several factors. These factors were the type of fermentation medium, the incubation period, the produced specificity of secondary metabolite compounds and the level of resistance of the test bacteria.

The utilization of three different types of liquid fermentation medium in this study was initially intended to determine the most optimum medium in producing secondary metabolite compounds from BR-S1 (A) isolate endophytic molds. However, the test results indicated no secondary metabolite compounds produced from the three types of liquid fermentation medium. Initial allegations to the absence of secondary metabolite compounds produced were the physical condition of the environment

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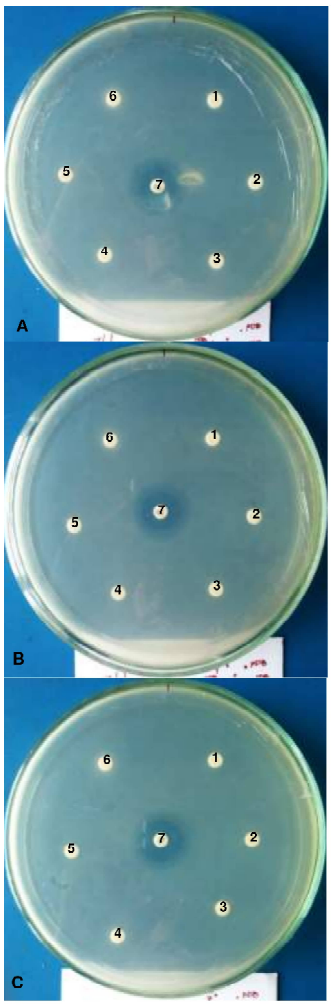

Figure 3. Antibacterial activity test results of BR-S1 (A) endophytic fungi isolate fungi against MRSA bacteria from three different types of liquid fermentation medium. A). 1st repetition; B). 2nd repetition, and C). 3rd repetition
Information figure 3:

1. Negative control of PDB extract
2. Negative control of CDB extract
3. Negative control of MEA extract
4. MEB Extract
5. CDB Extract
6. GDP Extract
7. Positive vancomycin control

(temperature) of incubation, too old endophytic mold isolates, and the composition of the fermentation medium. Previous research stated that one of the important factors influencing mold growth is the conditions of growth, if molds are in suitable conditions, the enzymatic expression to produce secondary metabolites is also maximum (4).

PDB, CDB, and MEB medium were synthetic/ semisynthetic medium selected for isolation, cultivation, and enumeration of molds or fungi in general. These three types of medium were able to support the growth of vegetative cells, mostly mold or fungi but these were unable to support the production of secondary metabolites. The composition of the fermentation medium greatly affected endophytic fungi in producing secondary metabolites (12). This was supported by the statement of (11) which stated that fermentation process by commercially available liquid medium often resulted on the yield of secondary metabolites which were limited. This was also supported by the findings of (7) study which revealed that the PDB medium was less able to stimulate the production of antibiotics which were able to inhibit both prokaryotic microbes of gram positive and eukaryotic.

Another factor which affected the absence of antibacterial activity ability from the extract of endophytic fungi isolate BR-S1 (A) was the incubation period selected to produce secondary metabolites. Each type of mold had a life cycle which varied in duration from the lag phase to the death phase. In this study, the incubation period used was 7 days and it was assumed that the 7-day growth of endophytic mold isolates of BR-S1 (A) had not reached to the stationary phase so that the production of secondary metabolite compounds was unavailable. This was in line with the statement of (10) which stated that the stationary phase was a phase in which the number of living and dead cells were relatively equal, the amount of nutrients begins to decrease so that it stimulated endophytic molds to produce secondary metabolites as a survival mechanism. Secondary metabolites were usually produced only in the stationary phase of growth.

Endophytic fungi isolate BR-S1 (A) was not able to inhibit/kill MRSA bacteria suspected because secondary metabolite compounds produced by these endophytic fungi obtained narrow specificity for certain types of bacteria and beyond MRSA bacteria type. In addition, it was also possible that MRSA bacteria had high resistance to secondary metabolites produced by endophytic mold isolates BR-S1 (A). This was in line with the statement by (9) which stated that each endophytic fungi was able to produce secondary metabolites which varied with different specificity and effectiveness. MRSA bacteria were Gram positive bacteria with a cell wall structure composed of layers of peptidoglycan, polysaccharides, and theatricic acid. These three cell wall components were covalently bonded to produce large and sophisticated cell walls which affected the antibacterial compounds tested could not penetrate the cell wall and did not enter the cell.

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4. Conclusion

Based on the results of the research and discussion above, several conclusions can be drawn as follows:

1. Three types of commercial medium,PDB, CDB and MEB medium, cannot be utilized as a fermentation medium for endophytic fungi isolates BR-S1 (A) in the production of bacterial compounds
2. Three types of PDB, CDB and MEBcommercial mediumare not optimum as a fermentation medium for endophytic fungi isolates BR-S1 (A) in the production of antibacterial compounds
3. Antibacterial compound extract of endophytic fungi isolate BR-S1 (A) results in the three types fermentation process of PDB, CDB, and MEBcommercial media are ineffective in inhibiting or killing MRSA bacteria

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Conflict of Interest

The authors have no conflicts of interest to declare. All co-authors have seen and agree with the contents of the manuscript and there is no financial interest to report. We certify that the submission is original work and is not under review at any other publication.

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Phytochemical Investigation, Cytotoxicity and Anti-Diabetic Activity of Whole Fresh and Dry Ethanolic Extracts of Sudanese *Portulaca quadrifida*

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Abstract

Historically, natural products have been used since ancient times and in folk medicine for the treatment of many diseases and illnesses. Portulacaquadrifida popularly called Chicken-weed belongto family Portulacaceae. In Sudanese folk medicine, this plant has been used to treat diabetes, and as a poultice to treat neuralgia in herpes zoster. Pharmacological activities of P. quadrifida include anti-microbial, anti hyperglycemic, antioxidant,anti-inflammatory, anti- nociceptive, anti convulsion properties, anti epileptic and anti cancer activity.In this study the phytochemical, Cytotoxicity and anti-diabetic activity of whole fresh and dry ethanolic extracts of Sudanese P. quadrifida L were investigated. The results showedhigh content of tannin 28.07 - 13.68 ppm for fresh and dry samples respectively. The P. quadrifidaextracts were found to be rich source of vitamin C (1.16 - 1.76 ppm) for fresh and dry respectively. In the cytotoxicity test using Microculture tetrazolium (MTT) assay, the IC50 values were 858 and 155.3092 ppm for dry and fresh samples extracts respectively. The Alpha amylase inhibition of both extracts showed high activity with inhibition percentage 95 and 91 % for dry and fresh extracts respectively at concentration 1000 ppm compared to the positive control (Acarbose).

Key words : Portulacaquadrifida, Tannin,Cytotoxicity, Anti-diabetic activity, Sudan.

1. Introduction

Historically, natural products have been used since ancient times and in folk for the treatment of many diseases and illnesses1.The first records that Plants were the basis of sophisticated traditional medicine system written on clay tablets in cuneiform, are from Mesopotamia and date from about 2600 B.C?2. During the 17th and 18th centuries recognition of plant use derived medicines grew rapidly3.The continuous and standing people's interest in medicinal plants has brought about today's modern fashion of medicinal plant processing and usage 4.Many people especially in developing countries resort to using medicinal plants and herbs instead of medicines due to the worse economic conditions and high prices of medicines. One of these medicinal herbs is Portulacaquadrifida popularly called Chickenweed,it is an annual much branched mat-forming, succulent species in the family Portulacaceae5.P. quadrifida stem is succulent, diffuse, and purple in color at maturity, less than a millimeter in diameter and up to 50 cm; rooting at the nodes6. The leaves are edible, frequently used in salads.Different parts are also used for various curative purposes. P. quadrifida have similar medicinal uses, but less widely used as P. oleracea7.P. quadrifidadistributed in Africa and tropical Asia; introduced into the warmer areas of the Americas, not existed in Australia5. The chemical constituents of P. quadrifida containing alkaloids, flavonoids, triterpenoids, glycosides, carbohydrates, tannins, amino acids and saponins8.P.quadrifidawas used traditionally to treaturinary discharges, inflammations, asthma, cough, and ulcers; a poultice of the plant is applied in abdominal complaints and hemorrhoids9. In Sudanese folk medicine, P.quadrifidais used to treat diabetes, and as poultice to treat neuralgia in herpes zoster. Thepharmacological activities of P.quadrifidaincludeantimicrobial, anti hyperglycemic, antioxidant10,anti-inflammatory, anti nociceptive activity11,anti convulsion;anti epileptic12and anti-cancer activities13 have been revealed in several studies.

2. Materials and Methods

Samples preparation and extraction procedure

P. quadrifida plant was collected directly from thefieldin Khartoum, Sudan. The plant was identified and authenticated by the herbarium unit of the Medicinal and

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Aromatic plants Institute Research Center. After washed by running tap water,samples were divided into two groups, Whole plant fresh and whole plant dry (air dried at room temperature). Both samples were extracted with absolute ethanol at room temperature for 72 hours. After filtration by Whatman filter paper Number 4,all samples were allowed to dryness.

Total Tannin Content (TTC)

The total tannin content of the extracts was determined as described byShanmukhaet al.14. About 1ml of Fecl3 (1%)and 1ml of K3Fe(CN)6(1%) were added to 1 ml of each extract (1mg/ml) and completed the volume to 10 ml with distilled water. Mixtures were shaken and left to stand at room temperature for 15 minutes and the UV-VIS absorbance was measured at 510 nm. Standard curve of tannic acid was used to quantify theP. quadrifida TTC. The standard curve was obtained by preparing set of 5 dilutions 100, 200, 300, 400, and 500 ppm tannic acid solutions from 1000 ppm stock solution. Absorbance was measured at 510 nm using Shimadzu spectrophotometer.

Quantitative determination of Ascorbic Acid (Vitamin C) using HPLC

Ascorbic acid content in the P. quadrifida extracts was determined by HPLC chromatographic method15 performed on a HPLCShimadzu, ODS-3 column (6 mm x 150) reversed phase matrix (5 ?m) and elution was carried out in a gradient system with0.1% (v/v) acetic acid :(95:5%)methanolwith 1.0 ml/min flow rate under ambient temperature. The volume of injection was 20 ?land the UV detector was set at 254 nm.Ascorbic acid standard solutions was prepared in concentration of 1, 10, 100 ppm and the standard curve was obtained.

Cytotoxicityusing MTT assay

The experiment was performed according to method described by Berridge et al16. Vero cells were cultured in a 96 -well plate for overnight CO2 environment at 37°C. Supernatant was removed, and 20 µl of serially diluted extracts (range from 0.01 to 100 µg/ml) and 80 µl complete medium DMEM supplemented with 5% (v/v) fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 µl/ml) were added to each well. After incubation, the culture medium was aspirated carefully and 50 µl of 3-(4, 5-dimethylthiazol) -2, 5diphenyle-tatrazolium bromide (MTT) solution (2 mg/ml PBS) was added to each well and further incubated for 4 hours. MTT solution was aspirated. The plate was agitated at room temperature for 15 min then read at 540 nm by using micro-plate readers. The optical density was measured at 540 nm and the percentage of viable cells was calculated as relative ratio of optical densities.

In vitro anti-diabetic activity

Serial concentrations of test samples (100-1000?g/ml) and standard drug (Acarbose) were prepared.About 500 ?l of each prepared solutions were transferred into another tests tube containing ? -amylase (0.5mg/ml) in 500 ?l of 0.20 mM phosphate buffer (pH 6.9) solution and incubated at room temperature for 10 min. After these, 500 ?l of 1% starch solution in 0.02 M sodium phosphate buffer (pH 6.9) were added to each tube and incubated at room temperature for 10 min. The reaction was stopped with 1.0 ml of 3,5 dinitrosalicylic acid (DNS)colour reagent. The test tubes were incubated in a boiling water bath at 100 0C for 5 min, cooled to room temperature. The reaction mixture was then diluted after adding 10 ml distilled water and absorbance was measured at 540 nm by Shimadzu Spectrophotometer UV double beam.17

Calculation of Inhibitory Concentration (IC50)

The concentration of the plant extracts required to inhibit 50% of the ?-amylase enzyme (IC50) were Calculated by using of Microsoft office Excel 2007. Inhibitionpercentage (%) was calculated by: % = (Ac-As)/Ac X 100

Where : Ac; is the absorbance of the control and AS;is the absorbance of the sample.

3. Results and Discussion

Total Tannin Content (TTC)

Total tannin content was expressed as milligram of tannic acid equivalent per gram. From the tannic acid standard curve(Figure 1) the TTC content was calculated.

The total tannin content of whole fresh and whole dry samples ethanolic extracts ofP. quadrifidawas evaluated. The highest content was found in fresh sample

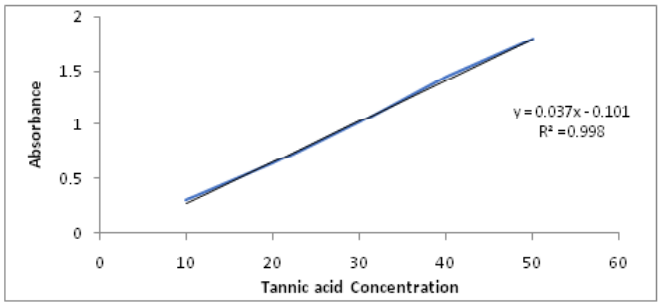

Figure 1. Tannic acid standard curve

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extract (28.07 ppm) followed by dry sample extract (13.68 ppm). The tannin compounds are widely distributed in many species of plants. Determination of the preliminary phytochemicals of P. quadrifida in several solvents demonstrates the presence of tannin in petroleum ether, chloroform and ethanol extract18.Severalstudies confirmed that the tannins exhibit antioxidant, antimicrobial, anti-inflammatory, antidiarrheal, and for heal burns and treat other diseases.19

Quantitative determination of ascorbic acid (vitamin C)

Ascorbic acid content in whole fresh and dryP. quadrifidaextract was determined using HPLC from the Ascorbic acid standard calibration curveand results are presented in Table 1. The fresh and dried whole plant extracts were found to be rich source of vitamin C. Both extracts showed comparable results of vitamin C determination (Figure 2,3,4).

Table 1. Ascorbic acid Content in whole fresh and dry extracts of P. *quadrifida*

Extract	Ascorbic acid Content (ppm)
Fresh P. *quadrifida*	1.160
Dry P. *quadrifida*	1.760

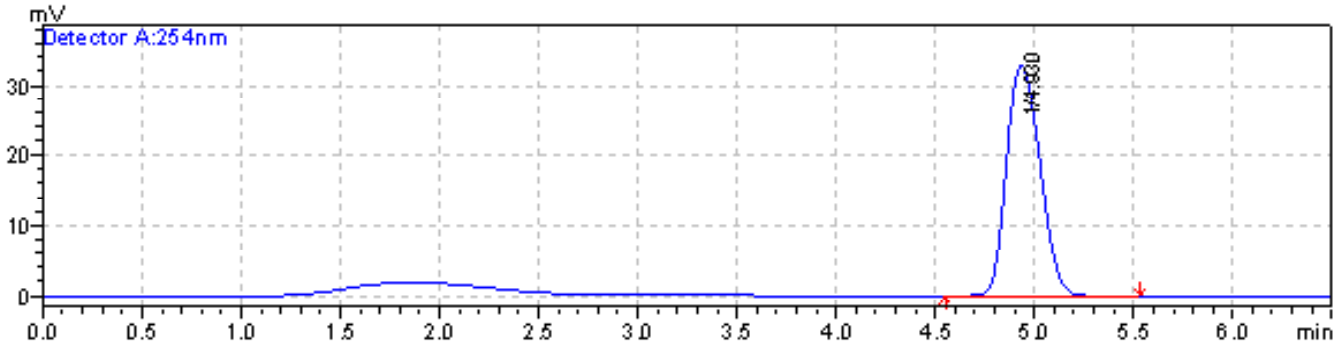

Figure 2. Spectrumof standard *ascorbic* acid

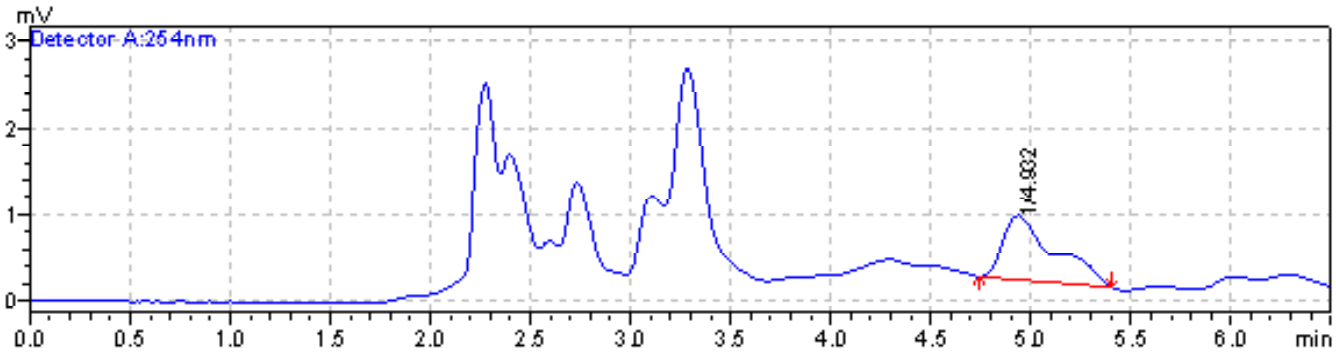

Figure 3. Spectrum of ascorbic acid in freshP. *quadrifida* extract

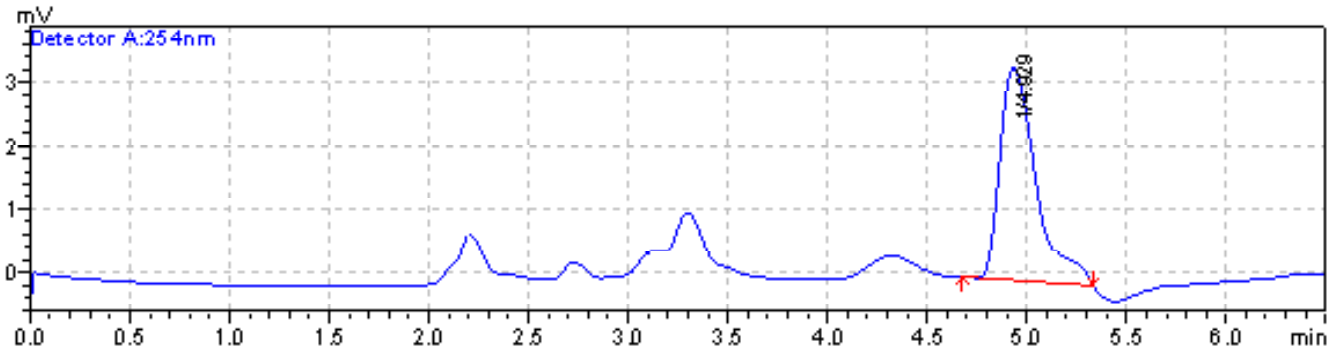

Figure 4. Spectrum of ascorbic acid in dry P. *quadrifida* extract

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MTT assay

Cytotoxicityof ethanolic extract of whole dry and fresh P. quadrifida at different concentrations (500, 125 and 62.5 ppm) was evaluated against normal Vero cell using MTT assay, results are presented in Table 2. The toxicity of both extracts was concentration dependent. The extract of dry P. quadrifidadisplayed low toxicity effect against Vero cells, while the fresh P. quadrifida extract showed high toxicity with highest inhibitory percentage (92.09 and 73.35 %) at concentration 500 and 125 ppm respectively.The IC50 value was calculated and the results were 858 and 155.3092 ppm for dry and fresh P. quadrifida sample extracts respectively. Extracts which revealed IC50<90 ppm were considered toxic20. Thus, it was clear both extracts of P. quadrifida were safe.

Table 2. Cytotoxicity of P. *quadrifidaextracts*

Sample	Concentration	Inhibition %	IC_{50}(ppm)
Whole Dry	62.5	-22.4476	858.7197
	125	-18.8811	
	500	33.84615	
Whole Fresh	62.5	-12.8671	155.3092
	125	73.35664	
	500	92.0979	

In vitro antidiabetic activity

Medicinal plants play an important role in the management of diabetes mellitus especially in developing countries. Moreover, during the past few years some of the new bioactive drugs isolated from plants showed anti-diabetic activity used in clinical therapy.21 In anti-diabetic activity evaluation by alpha amylase inhibition, both extracts showed highest activity with inhibition percentage 95 and 91 % for dry and fresh extracts respectively. Results are presented in Table 3. The anti-diabetic drugs from plants in current clinical use are preferred mainly due to lesser side effects and low cost.

Results indicated that both fresh and dried P.quadrifida possessed anti-diabetic activity,besides stronger activity was observed in the fresh herb. These findings provided evidence for the application and development of fresh extract in the treatment of diabetes mellitus18. Comparing the results from the two extracts, the whole dry and the whole fresh ofP.quadrifida shows comparable values of vitamin C content and anti- diabetic activity. The whole dry extract showed lesser toxicity than whole fresh extract while the fresh whole extract showed measurable higher tannin content compared to the dry whole extract.

Table 3. α-amylase inhibition percentage of P.*quadrifidaextracts*

Sample	**Inhibition %**			**IC_{50} (ppm)**
	250 ppm	**500 ppm**	**1000 ppm**	
Dry *P.quadrifida*	58.28274	87.33738	95.4033	179.5797
Fresh *P.quadrifida*	58.54293	85.16912	91.4137	173.0966
Control +ve (Acarbose)		90.80659 %		

4. Conclusion

Generally, natural sources are rich in chemical constituent that observe beneficial medical uses. P.quadrifida is one of these plants that show medicinal uses but less widely investigated. The high Tannin, vitamin C levels,less toxicity, and anti-diabetic activity for both whole fresh and the whole dry extracts of Sudanese P.quadrifida were demonstrated the uses of P.quadrifida as traditional medicine.Farther investigations for other traditional uses of P.quadrifidamust be examined. The results of this study augment the importance of the use of natural plant and natural product as a source of medicinal agent. Further investigations are important for the identification of active principles to development new drugs against various diseases.

Conflict of interests

Authors declareno conflict of interests.

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Analysis of Prednisone in Indonesian Uric Acid Herbs Using High Performance Liquid Chromatography

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Abstract

The problem of adulteration of herbal medicines product with active pharmaceutical ingredients (API) has existed for years in Indonesia. According to the government's rules, it is not allowed to add API to traditional herbal medicine. Uric acid herbs are one of the most popular herbal medicine products. Prednisone is one of the corticosteroids that has been reported to be detected in herbal products. The purpose of this study is to identify prednisone in uric acid herbs using High Performance Liquid Chromatography (HPLC). The stationary phase used in this study was C18 Puroshper®STAR RP-18e LiChroCART® column (250 - 4.6 mm; 5 µm i.d.), while the mobile phase was methanol : water (60 : 40 v/v). The mobile phase flow rate was set at 1 ml/min. Prednisone in the sample was detected at wavelength 243 nm using a UV detector. Validation methods in this study consisted of precision, linearity, the limit of detection (LOD), the limit of quantitation (LOQ), and accuracy. The precision is indicated by the relative standard deviation (RSD) value of 0.33% (<2%). The correlation coefficient value (r) of 0.9955 obtained from the prednisone calibration curve shows the method's linearity. The recovery values of 100.11 ± 0.82 % indicates the accuracy of the method that meets the requirements. The LOD and LOQ values were 2.96 and 9.85 µg/ml, respectively. Method validation parameters have been proven that meet the requirements. The HPLC method can be used to analyze prednisone in uric acid herb samples. The application of the method for analysis of eight herbal products taken from the market shows that prednisone was detected in two products.

Key words : prednisone · uric acid· herbs · HPLC

1. Introduction

Jamu / Herbs is a herbal preparation, an Indonesian traditional medicine. Herbs that produced by the manufacturer has to be given label JAMU and a unique logo of jamu in the package. Based on Indonesia's regulations, traditional medicines are prohibited from containing isolated or synthetic medicinal chemicals. However, the existence of active pharmaceutical in herbal products is still found (1).

Steroid, including prednisone, was one of the most frequent adulterants. An examination of distributed jamu thus becomes an important issue to prevent harmful side effects due to adulterated herbal medicine.

Some methods reported have been published to analyze prednisone in medicinal herbal medicine products such as Thin Layer Chromatography (2); Solid Phase Extraction-High Performance Liquid Chromatography/ HPLC (3);Ultra-Performance Liquid Chromatography-Mass Spectrometry (4), and Gas Chromatography-Mass Spectrometry (5). The purpose of this study is to identify prednisone in uric acid herbs using simple High Performance Liquid Chromatography (HPLC) method with an ultraviolet detector.

2. Materials and Methods

The Separation was carried out on a set of HPLC instruments (Shimadzu Prominence-i LC-2030C) equipped with Puroshper®STAR C18 columns (250 mm x 4.6 mm i.d. 5µm). The absorption spectrum was made using a Shimadzu 1800 UV-VIS Spectrophotometer. The different brands of uric acid herbs were taken from several shops in Purwokerto, Central Java, Indonesia. Sample from eight different companies were coded as A, B, C, D, E, F, G, and H.

***Preparation of the mobile phase* :** The mobile phase was made from a mixture of methanol and water at a ratio of 60:40 v/v. The mixture is then filtered and sonicated for 20 minutes.

***Preparation of standard solution* :** The prednisone reference standard was carefully weighed as much as 10

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mg and put in a 10 ml volumetric flask, then dissolved with a mobile phase quantitatively to obtain a 1000 µg/ml solution. From there, the solution was pipetted 1.0 ml and put into a 10 ml volumetric flask, and then diluted quantitatively with a mobile phase so that a solution of 100 µg / ml is obtained.

Determination of the maximum wavelength of prednisone :The 10 µg/ml prednisone reference solution was scanned at a wavelength of 200 - 400 nm using a UV-Vis spectrophotometer. The wavelength with maximum absorption was determined from the spectrum obtained.

***Optimization of the composition of the mobile phase*:** Some mobile phase compositions used are: buffer pH 4 and methanol (80:20 v/v); buffer pH 4 and acetonitrile (80:20 v/v); methanol and water (50:50 v/v); and methanol and water (60:40 v/v).

***System suitability test* :** A prednisone standard solution of 25 µg/ml was prepared 6 times and then injected into HPLC with a volume of 20 µl. The mobile phase flow rate was set at 1.0 ml/min. Retention time, peak area, and tailing factor were recorded. Then the average, SD, and RSD were calculated.

***Preparation of prednisone calibration curve* :** Prednisone solution in the mobile phase with a concentration of 10; 15; 20; 25; 30 and 35 µg/ ml were prepared. Each solution was filtered and sonicated for 10 minutes. Then each of them was injected into the HPLC. The peak area shown on the chromatogram was recorded. The plot between prednisone concentration and peak area was made.

***Validation of analytical methods* :**The validation parameters of the tested analytical methods include selectivity, linearity, the limit of detection and limit of quantitation, precision, and accuracy. Validation was done according to ICH guidelines (6).

***Determination of prednisone in the sample* :** Uric acid herbs samples of brands A, B, C, D, E, F, G, and H were carefully weighed 150; 4500; 75; 40; 560; 187.5; 750; and 2500 mg, respectively. Then, put in a 10 mL volumetric flask, dissolved with the mobile phase, then filtered and sonicated for 20 minutes. The solution was injected into the HPLC. The determination was carried out in triplicate.

3. Results and Discussion

***The maximum wavelength of prednisone* :** Based on the UV absorption spectrum of prednisone, a wavelenght of 243 nm was used for the detection of the drug in this HPLC method.

***The optimum composition of the mobile phase* :** In this study, prednisone analysis in uric acid herbs was carried out by HPLC with a stationary phase of RP-18. Based on the results of the optimization of the mobile phase, the most optimal separation is produced by a mixture of the mobile phase of methanol : water (60:40 v/v).

***System suitability* :** System suitability is an integral part of the analysis procedure. The test is based on the concept that equipment, electronics, analysis procedure, and the samples to be analyzed are the whole system so that they can be evaluated (6). Table 1 shows the results of the system suitability test results. From repeated injection of prednisone solution in HPLC, the RSD values for the parameters of retention time, peak area, and tailing factor are <1.0%, respectively. The tailing factor of 1.639 shows the shape of the peak that meets the criteria of asymmetric aspects because its value is less than 2.0 (7). The system suitability test results show that the conditions used to determine prednisone levels have a good system suitability based on the RSD value <2% (7).

Table 1 : System Suitability

Injection No.	Retention time (min)	Area	*Tailing factor*
1	6.567	822,764	1.615
2	6.517	822,175	1.643
3	6.615	827,412	1.626
4	6.610	827,821	1.645
5	6.609	823,670	1.651
6	6.611	828,167	1.655
Average	6.588	825,334.83	1.639
SD	0.039	2,752.53	0.015
RSD (%)	0.594	0.33	0.944

***Prednisone calibration curve* :** Figure 1 shows a prednisone calibration curve made in the range of 10 - 35 µg/mL. The calibration curve obtained is used to calculate prednisone levels in the sample.

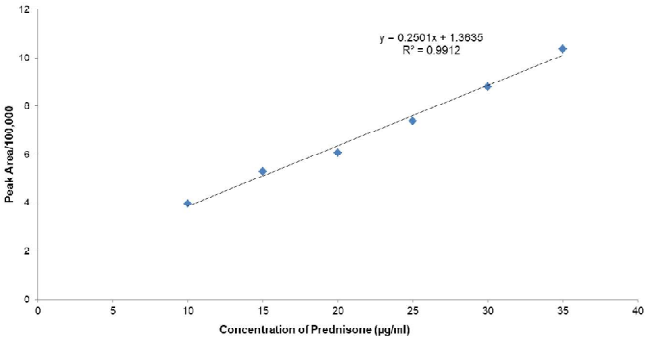

Fig. 1. Prednisone calibration curve

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Table 2 : Method validation result

Parameter	Result
Retention time	6.588 ± 0.039 minute
Linearity	r = 0.9955
Range	10-35 µg/mL
LOD	2.95 µg/mL
LOQ	9.85 µg/mL
Precision	RSD = 0.33 %.

Table 3: Accuracy of Prednisone Quantitative Analysis Method in Herbs

Sample	Standard added (µg/mL)	Standard found (µg/mL)	Recovery (%)	SD	RSD (%)
A	35	32.41	92.26	2.24	2.42
B	35	36.06	100.94	1.70	1.65
C	35	35.01	100.04	0.07	0.07
D	35	33.59	95.96	0.95	0.99
E	35	35.78	102.24	0.18	0.18
F	35	35.82	102.34	0.40	0.39
G	35	35.80	102.27	0.36	0.35
H	35	36.07	103.05	0.66	0.65

Validation of analytical methods **:** Figure 2 shows the selectivity of the HPLC method that meets the criteria.

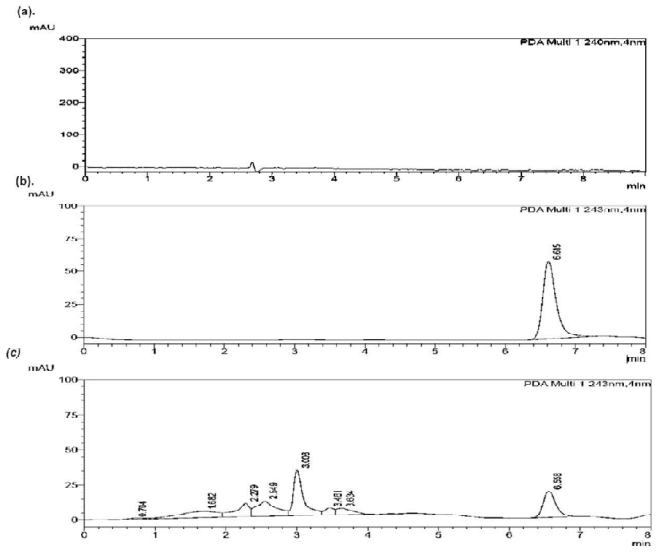

Fig. 2. Chromatogram of (a) Blank; (b). Prednisone reference; and (c) Uric acid herbs product.

Around the prednisone peak, which is retention time around 6, 5 minutes, there is no potential for interference from other components of the mobile phase or sample matrix.Table 2 shows the results of the analysis method validation. The prednisone retention time was 6.588 ± 0.039 minutes. Linearity is indicated by the correlation coefficient close to 1.0 (r=0.9955). The linear relationship between concentration and peak area was proven in the range of 10-35 µg/mL.The detection limit and the quantitation limit were determined by mathematical calculation of the calibration curve. The detection limit and the limit of quantitation are 2.95 µg/mL and 9.85 µg/mL, respectively. Precision is indicated by the RSD value of less than 2.0 %. Table 3 shows the accuracy of the HPLC method for Prednisone analysis in uric acid herbs. The recovery value is from 92.26 to 103.05% (average recovery 100.11 ± 0.82 %). The accuracy criterion for the analysis method is that the average recovery value is 100 ± 2% (7).

Table 4 : The results of the analysis of prednisone in uric acid herb products

Sample	Prednisone content (µg/mg)
A	n.d.
B	n.d.
C	21.76 ± 0.44
D	50.07 ± 0.30
E	n.d.
F	n.d.
G	n.d.
H	n.d

n.d. = not detected

The results of the analysis of prednisone in uric acid herb products **:** The results of the analysis of uric acid herbs samples shown in Table 4 shows that the adulteration of active pharmaceutical ingredients, especially prednisone, is still found in two products taken from the market in Indonesia. The presence of adulteration in this study is an addition to the previous findings. In previous studies it has been reported that in antidiabetic jamu still found the active pharmaceutical ingredients of glibenclamide (8). In "kuat lelaki" jamu found sildenafil as an adulterant (9), whereas in "pegal linu" jamu there was no paracetamol detected (10). Another study also reported that sibutramine as adulterant was detected in herbal slimming products collected from the market in Depok City, West Java, Indonesia (11).

The active pharmaceutical ingredients are also found in various herbal supplement products in several countries

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such as the presence of cyproheptadine and dexamethasone in weight gain product in Iran (12); sildenafil, tadalafil, and vardenafil hydrochloride in herbal medicine and food samples collected in Sultanate of Oman (13).

4. Conclusion

The HPLC method was successful in clearly identifying and quantifying prednisone present in uric acid herbs. From eight herbs, showed that two samples (25%) confirmed the presence of prednisone as an adulterant. This also calls for a thorough focus on making the regulation systems for this jamu stricter. The regulations related to licensing and labeling of jamu should be as strong as to ensure 100 % product integrity.

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Conflict of Interest

The authors declare no conflict of interest.

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An Analysis of Rat Meat with FTIR and GC / MS for Halal Authentication

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Abstract

Fourier Transform Infra Red (FTIR) and Gas Chromatography Mass Spectrophotometry (GCMS) method were developed to determine rat fat which is extracted from rat meat. Fat was extracted with methanol and chloroform as a solvent. Derivatization process was conducted to convert fat into a methyl ester form using sodium methoxide and boron trifluoride. GCMS analysis was used to identify the fatty acid composition from rat fat. The FTIR spectral bands correlated with bovine, pork, chicken and rat fat were scanned, interpreted, and identified. Qualitative differences between FTIR spectra were proposed as a basis tools for differentiating between rat fat and other fat. Principal Component Analysis (PCA) at combining wavenumber regions of 1250-1100 cm-1 and 3010-2850 cm-1 was capable of distinguishing rat meat from other meat. GCMS chromatogram showed the fatty acid composition in rat meat which five major compounds were hexadecenoic acid, 9,12-octadecadienoic acid, 9-hexedecenoic acid, tetradecanoic acid and 7-hexadecanoic acid. PCA based on fatty acid composition can distinguish rat meat from other meat.

Key words : Rat meat · FTIR · GCMS ·

1. Introduction

A muslim is majority population in Indonesia, whose must consume halal food products. Thus, it is important to ensure the halals of food products. Adulteration non halal meat in food product become increase and some food products are found to have been adulterated with non halal ingredient, such as rat meat in meatball (1,2).

Meatballs are a popular food in Indonesia where the main component is meat. The meat can be from beef, chicken, or fish. Some food manufacturers replace halal meat with non halal meat such as rat, because it is easier to get and cheaper. The aim to reduce the cost. However, it is unfavorable for consumers and harmful to health because rat can cause some diseases like Salmonellosis, Leptospirosis and Plague (1).

Non halal component in food products can be analyzed with several methods, such as Gas Chromatography-Mass Spectrometry/GCMS (3,4), Fourier Transform Infra Red/ FTIR (5,6,8), Liquid Chromatography/HPLC (9) and Polymerase Chain Reaction/PCR (7). FTIR method is fast and consistent method, even in low analyte concentration (10), non-destructive, sensitive, and does not require complicated sample preparation (11).

However, the FTIR method has limitations, that is it cannot certainly identify the content type of the sample's each fatty acid component (8). Fatty acid analysis can be done by GCMS method. Fatty acid composition of meat can be used for distinguishing rat meat from other meat. The fatty acid composition is determined as methyl ester. The purpose of this research is to identify rat meat in food products by using FTIR and GCMS methods.

2. Materials and Methods

Lipid Extraction :

Black rats were obtained from local farm in Banyumas regency Indonesia. Bovine, pork and chicken meat was obtained from local market in Banyumas Indonesia. Fat was extracted using chloroform: methanol by Bligh & Dyer methods.

GC-MS Analysis :

Fat was hydrolyzed with alkaline to produce fatty acid, and followed with methyl esterification. Transesterification performed by the BF_3-MeOH method to form fatty acid methyl ester. Approximately 50 µL oil samples were added with 1.0 mL n-hexane and 200 µL 0.2 N $NaOCH_3$ solutions and heated at 60°C for 10 min. Then, the mixture was added to 1.5 mL BF_3-methanol reagent, and heated at 60°C for 10 min. After it was cool, 1 mL of saturated NaCl solution was added, and shake. The resulting hexane layer was used as a sample solution for GC-MS. Subsequently, 1 µL of the clear supernatant was taken and injected into a GC-MS (Shimadzu QP2010, Shimadzu Corp., Tokyo, Japan). The column used is a

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SH-Rxi-5Sil MS (5% diphenyl/95% dimethyl polysiloxane) capillary column (30 m x 0.25 mm ID, 0.25 µm film thickness). Helium was used as the carrier gas at flow rates of 1.0 mL/min. The injector temperature was 280°C. The oven temperature was set at 100°C for 5 min, increased to 240°C at a rate of 4°C/min and held at the final temperature for 30 min. The GC-MS operation was controlled by Lab Solution software. MS spectra were obtained in wide range of m/z 10- 500. FAME peaks were identified by comparing their retention time with the FAME standard and similarity index (SI more than 90%).

***Fat analysis using FTIR* :**

Fat from each meat was dropped on the ATR crystal, which was placed in a controlled temperature (20°C) as much as 1 drop. Then, the fat was scanned for 32 times at the wave number of 4000-650 cm^{-1} with a resolution of 4 cm^{-1} and was recorded in the form of absorbance. FTIR spectra were analyzed using chemometrics in the form of PCA using Horizon MB software.

3. Results and Discussion

Fatty acid composition was identified and measured with gas chromatography with mass spectrometry (GC MS) which present in the lipid extracted from rats and other meat. Saponification with alkaline and followed by BF^3-catalyzed methylation were used to form fatty acid methyl ester. Peak identification of fatty acids methyl ester in the analyzed samples was conducted by comparing the retention time and molecular mass of mass spectra of standard mass spectra, which were obtained from library (Wiley9.lib) of the GCMS instrument and also confirmed by comparing the mass spectrometric fragmentation pattern with the standard.

Composition of fatty acid can be used to differentiate rat fat from other species. GCMS chromatogram at figure 1 revealed that hexadecanoic acid has the highest level fatty acid from rat fat. The other major constituents are; 9,12-octadecadienoic acid (tr 34.424 min); 9-hexadecenoic

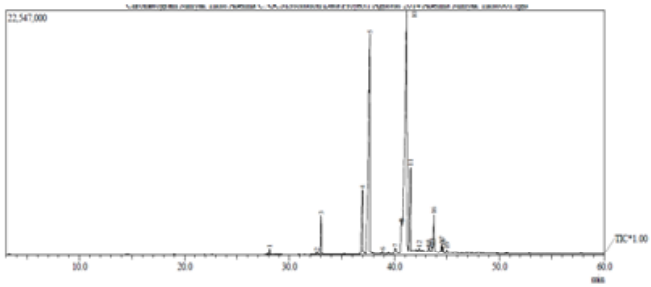

Figure 1. Rat's fat GC chromatogram

Table 1: Fatty Acid Compositions of Lipid Extracted from Bovine, Rat, Chicken and Pork Obtained By GC-MS Method

Fatty Acid	Fatty Acid Percentage (%)			
	Rat	Bovine	Chicken	Pork
Dodecanoic Acid	0	0	0.07	0.2
pentadecanoic acid	0.2	0.61	0.06	0.09
tetradecanoic acid	1.79	3.18	0.56	1.82
9-octadecenoic acid)	0	24.52	46.57	37.62
cis-9-tetradecenoic acid	0.05	1.32	0	0
7-hexadecanoic acid	1.31	0.1	0.28	0.42
hexadecanoic acid	25.09	23.75	24.62	24.19
9-hexedecenoic acid	1.79	4.64	3.7	2.27
octadecanoic acid	0.32	12.75	7.74	13.15
heptadecanoic acid	0.29	1.17	0.1	0.44
cis-10-heptadecenoic acid	0.1	1.02	0.04	0.25
9,12-octadecadienoic	16.11	1.57	15.59	17.36
5,8,11,14-eicostate	0	0	0	0.22
6,9,12-octadecadienoic	0	0	0	0.13
eicosenoic acid	0.15	0.07	0.06	0.18
methyl eicosanoic acid	0.56	0	0	0
10-nonadecenoic acid	0	0.24	0	0
11,13-eicosadienoic	0.06	0	0	0.45
11-octadecenoic acid	0	47.43	0	0

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acid (tr 29.594 min); tetradecanoic acid (tr 24.64 min); and 7-hexadecanoic acid (tr 29.140 min). From Table 1 it can be seen that rat has a larger percentage of saturated fatty acids than beef, chicken and pork. The differences in the degree of unsaturated fatty acid in animal fats could be due to the individual fatty acid distribution pattern (12). The presence of methyleicosanoic acid is found in lipid extracted from rats, but it is not found in bovine. In contrast, 11- octadecenoic acid (47.30%) exists only in lipid extracted from bovine. Hexadecanoic acid is found to be approximately equal in all analyzed lipid types. The quantity of 9.12-Octadecadienoic acid in bovine (1.57%) is much lower than in rats (16.11 %). The fatty acid composition of lipid extracted from rats were unique compared to bovine, chicken and pork. From the GC-MS analysis, it is found that the major constituents of lipid extracted form rats were fatty acids with chain lengths of 15 to 21 carbon atoms (mainly C17 and C19). PCA score plot in Figure 2 showed that fatty acid composition can distinguish rat fat from other fat. Rat fat is located at different quadran from other fat.

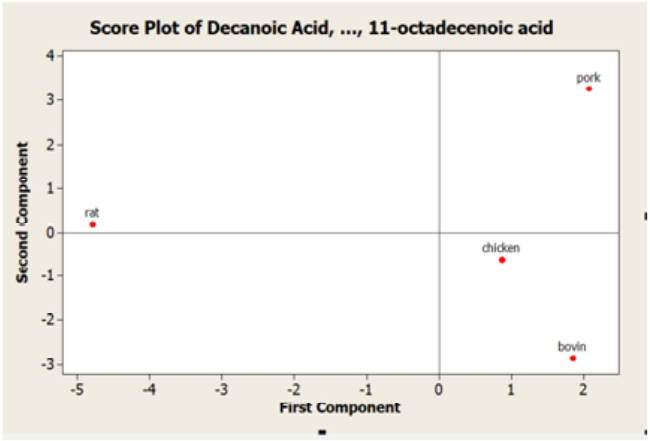

Figure 2. PCA Score Plot from rat, chicken, pork and bovine fat based on fatty acid composition

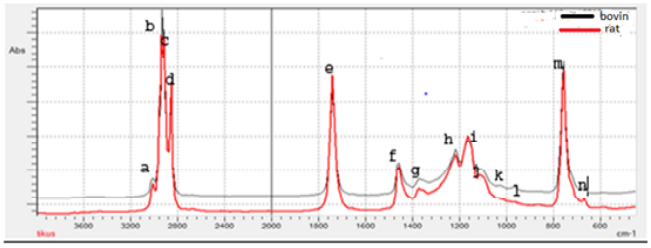

Figure 3. FTIR spectrum rat and bovin fat

FTIR analysis was conducted based on the differences between the functional groups of fat from rat, chicken, pork and bovine meat, which were measured at wave number 4000-650 cm^{-1}. Figure 3 shows that the difference of FTIR spectrum between rat and bovine fat is a typical

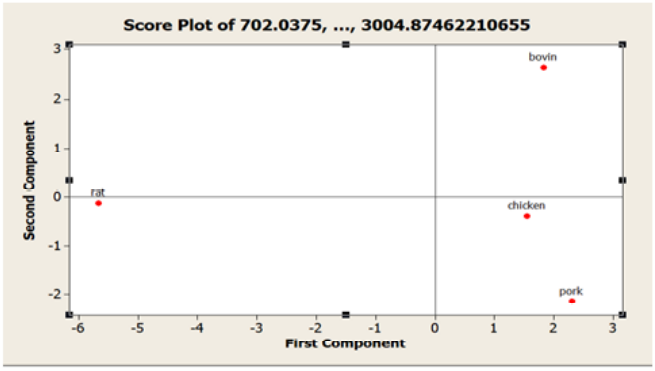

Figure 4. PCA Score Plot From Rat, Chicken, Pork And Bovine Fat at wavenumber 1250-1100 cm^{-1} and 3010-2850 cm^{-1}

peak at wave number 3010-2950 cm^{-1}. The absorption pattern at wave number 3008.95 cm^{-1} (peak a) for rat fat shows relatively higher peaks compared to bovine fat. The high peak of rat fat absorbance in the area shows the presence of unsaturated fatty acid content which contributes to the high absorbance value, namely the C-H stretching vibration area of the cis double bond.

Fingerprint area (1500-700 cm^{-1}) that is at wavenumber of 1118.71 cm^{-1} (i) shows the typical spectrum of rat fat that is the C-O stretching vibration area. The third point of difference is located in wavenumber 1026.13 cm^{-1} (k) and 972.12 cm^{-1} (l) in which this area does not show any absorption in the rat fat spectrum.

Figure 4 showed that PCA can be accomplished to classify between rat, pork, chicken and bovine fat. Rat fat stand far from others fat, chicken and pork stand at same quadrant which it showed they have similarity.

4. Conclusion

This study investigated application of GC-MS and FTIR to identify rat meat based on fat and fatty acid profile. The high constituents of lipid extracted from rats are 9-Octadecenoic acid; hexadecanoic acid; 9,12-octadecadienoic acid; octadecanoic acid; 9-hexadecenoic acid; tetradecanoic acid; and 7 hexadecanoic acid. The major constituents are fatty acids with chain lengths of 15 to 21 carbon atoms (mainly C17 and C19) and unsaturated fatty acid higher than saturated fatty acid. GCMS method can be used to authenticate rat meat with fatty acids content.

Application of multivariate statistical analysis such as PCA would be required to determine source of the origin. Hence, this study showed that fatty acid data allowed separation rat fat from other animal fats.

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The difference of rat and beef fat is located in wavenumber 1026.13 cm^{-1} (k) and 972.12 cm^{-1} (l) in which this area does not show any absorption in the rat fat spectrum. FTIR spectroscopy at wavenumber region 1250-1100 cm^{-1} and 3010-2850 cm^{-1} combined with chemometrics techniques can be used to determine rat meat.

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Conflict of Interest

The authors declare no conflict of interest.

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Epidemiological Studies of Schistomiasis in Bauchi Central Senatorial Zone, Nigeria

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Abstract

A twelve months Epidemiological Studies was conducted in Bauchi Central Senatorial Zone in 2016 to determine the prevalence, water contact activities, water quality and vector aspect of schistosomiasis in the study area. Six hundred 600 samples of each urines and stools were collected and examined microscopically for schistosomes eggs. The urine samples were examined using sedimentation method while the stool samples were examined using formol-ether concentration technique. Twelve 12(2%) out of the entire urine samples examined had eggs of Schistosoma haematobium and none of the stool samples were positive with the egg of any intestinal shistosomes. Two water bodies were randomly selected from each selected local governments for surveyed of the intermediate hosts (snails) of the parasite. The intermediate host were collected and examined for cercariae by exposing them to sunshine for 30 minutes in a beaker containing water and water samples were also collected for water quality studies such as Ph, temperature and dissolved oxygen. Four hundred and twenty two (422) snails were collected and examined. Out of it, only 21(4.9%) Bulinusglobosusshed cercariae and also the only vector of the parasite found in the area. Six hundred (600) questionnaires were distributed in order to determine the participants' knowledge and perception about the parasite, sex, age, water source, toilet facilities and their occupations. The infection rates by the parasite in different sexes is not statistically significant ($p>0.05$) while in different age groups, individuals using different water source, individuals using different types of toilet facilities and individual with different occupational groups were all statistically significant ($P<0.05$). The water quality seemed to have an effect on the infectivity of the snail vectors as out of the 422 snails collected and examined only 21(4.9%) snails were infected in the water with low pH value and high dissolved oxygen. From the results obtained, schistosomiasis is not endemic in the study area. Health education is recommended to maintain the non-endemic nature of the parasite in the study area.

Key words : Epidemiology, Schistosomiasis, Bulinusglobosus, Cercariae, Bauchi

1. Introduction

Schistosomiasis is a complex water-borne disease caused by blood-dwelling trematode worms of the genus Schistosoma and five species parasitizing humans include Schistosoma haematobium, Schistosoma mansoni, Schistosoma japonicum, Schistosoma intercalatum and Schistosoma mekongi. After malaria and intestinal helminthiasis, schistosomiasis is the third most devastating tropical disease in the world, being a major source of morbidity and mortality for developing countries in Africa, South America, the Caribbean, the Middle East, and Asia (28). It is estimated that 200 million people are infected, of which 120 million are symptomatic and 20 million have severe disease. In 74 countries, 600 million persons are at risk of infection (9,15) and the disease is principally for tropical and sub-tropical regions which found in South and Central America, Africa, Asia and South - East Asia. It is estimates suggest that 85% of all schistosomiasis cases are now in Subsaharan Africa (9) and centers for disease control and prevention in (2013) classified the disease as tropical neglected disease. The disease occurs in all the 36 state of Nigeria including the Federal Capital Territory (12). Nigeria is an endemic area with an estimated 11 million people infected (18). (90) estimated that 101.28 million people are at risk of infection in Nigeria while 25.83 million people are actually infected.

Snails are the intermediate host of the parasite and snail ecologists have tried to correlate snail distribution with physico chemical factors and to discover the ranges of these factors within which the snails thrive (33). Under natural conditions, snail are exposed to a range of varying and often interacting environmental factors which produce collective effect on them and it is usually difficult to separate the effect of any factor from others (4). It is well documented that intermediate host snail inhabit a wide range of natural habitats (7, 25, 26, 29, 31). Man made

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habitats such as irrigation canals, pools behind dams, ponds along roads and ditches (temporal habitats) may become rapidly inhabited by these intermediate host snails, thus contributing to disease transmission (2, 17, 24, 27). Therefore, understanding the ecology of the snail intermediate host, geographical distribution of the snail vectors borne disease and also their transmission process is very important in tackling the disease.

This study is aim at determining the prevalence of the parasite and other factors that influences the transmission of the parasite such as snail populations (intermediate host) and physio-chemical qualities of the water bodies, thereby providing adequate information that can be utilized in designing a suitable programmed for effective control of schistosomiasis in study area.

2. Materials and Methods

Study area

The study was conducted in Bauchi Central Senatorial Zone, Nigeria. Six local governments make up the zone out of the twenty local government of the state which includes Ningi, Warji, Ganjuwa, Misau, Dambam and Darazo. The zone occupies a total land of 15627km representing about 32.4% of the state's total land area. According to the 2006 census, the zone has a total population of 1448386. The zone has two distinctive vegetation namely Sudan and Sahel savannah. During the dry season, pools of the varying sizes (lakes, ponds, dams, ditches) are found in various parts of the zone which serve as a source of water for domestic, recreational, occupational and socio-cultural purposes (35).

Sampling techniques

A random sampling technique was employed to select three (3) local governments out of the six (6) local governments in the zone and two water sites in each of the selected local governments for snail collections. The sample size of 600 was determined using the formula describe by (19, 3) and the sample size of each local government was also determined by (21).

Sample collection

Each selected individual was given two containers for collection of stool and urine and a questionnaire. Before giving the containers, they were instructed on when and how to collect the samples. Snail and water samples were collected from the selected water bodies using long handed dip net to scoop at each site for 10 minutes which was adopted (16) to collect the snail and bottle for water samples.

Identification and examination of snails

The snails collected were separated and identified on the arrival to laboratory according to standard key described by (8). Each species were placed in a separate glass beaker bearing labels showing the location of collection; reference number and date of collection. 10 snails were placed in each beaker 500ml capacity. 100ml of water was added before exposing them to sun- light for 30 minutes to facilitate the shedding of cercariae by the snails. Then, the water in the snail containers were examined for cercariaeunder a dissecting microscope. Each snail in the containers that is positive with cercariae was separated and examined further, by placing each of them in a separate beaker. 10mls of water was added and exposed to sunlight for another 30 minutes. The water examined again for emergence.

Water sample analysis

Water samples were collected from the selected sites and taken to the laboratory and analyzed for temperature, Ph and dissolved oxygen. Temperature was determined using portable Hanna instruments Dist 5 EC/TDS/ Temperature Tester HI98311.o c, pH was determined using Hanna instrument pHep(R) pH Tester - HI98107 while dissolved oxygen was determined by Wicklers method.

Ethical clearance

Permission and consent was sought from the Bauchi state government (MOH/GEN/S/1409/1) before proceeding with the research after which the District heads of the selected local governments were approached for same purpose as well as the consent of the individual participants.

Data analysis

The data collected were subjected to Chi-square test as the relationships between two variables were compared and simple percentage. $p<0.05$ were use to determined the level of significance.

3. Results and Discussion

A total of 422 snails were collected from January to December, 2016 for the purpose of this study. During the study only Bulinusglobosus species were found in the area that can transmit the parasite and 21 (4.9%) out of Bulinusglobosus were infected with the infective stage of the parasite or shed cercariae. Table 1 showed the

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Table 1 : Prevalence of snails collected according to month during the study

Months	No of snail collected	No Infected (SNAIL) = Observed (O) (%)	infectivity rate
January	42	2	4.8
February	39	0	0
March	12	0	0
April	18	0	0
May	7	0	0
June	0	0	0
July	0	0	0
August	0	0	0
September	37	2	5.4
October	78	7	8.9
November	103	6	5.8
December	86	4	4.7
Total	422	21	4.9

X^2 calculated = 20.57; X^2 tabulated = 19.68, df = 11, P>0.05

number of snails collected and examined in each month during the study. The month of October had the highest infected snails with 8.9%, followed by November, September, January, and December with 5.8%, 5.4%, 4.8%, and 4.7% respectively while in February, March, April, May, June, July and August no was infected or snail shed cercariae. The infections rate among the snails collected during the study in relation to month were no significantly different at p>0.05. Also the prevalence rates of the parasite in different locations (local government). Was not significant at p<0.05. A total of 600 urine and stool samples each were collected in the three selected local government in zone. The samples collected in each of the local government are 225, 136 and 239 in Misau, Dambam and Ganjuwa respectively. Ganjuwa local government had the highest prevalence rate with 8(3.3%) followed by Misau and Dambam had the lowest with 3(1.3%) and 1(0.7%) respectively as show in table 2.

Table 2 : Prevalence of The Parasite in Three selected local government in the Zone.

Local Govt.	No Examined	No Infected	Prevalence (%)
Misau	225	3	1.3
Dambam	136	1	0.7
Ganjuwa	239	8	3.3
Total	600	12	2

X^2 Calculated = 3.83; X^2 Tabulated = 5.991, Df= 2, P>0.05

Table 3 shows the infection rate according to group age of the participants, out of all the age group examined 11-20 year age group recorded the highest prevalence rate with 9(5.2%) followed by 21-30, 31-40 with 2 (1.8%), 1 (0.9%) respectively and there was no infection in age group 4-10 and 41 - above. The infections rate among different age group was statistically significant (P<0.05). Also the infection rate according to gender, shows out of the 600 samples examined (420 male and 180 female). Female had the highest infection rate when compare with male counterpart with 4(2.2%) and 8(1.9%). The infection rate between the sexes was significantly different (P>0.05) as shown in table 4.

Table 3 : Prevalence of Schistosomiasis in the Different Age Groups of Inhabitants in Bauchi central senatorial zone.

Age	No Examined	No Infected	Prevalence (%)
4-10	130	0	0
11-20	172	9	5.2
21-30	113	2	1.8
31-40	103	1	0.9
41- above	82	0	0
Total	600	12	2

X^2 calculated = 27.97; X^2 tabulated = 9.488, df= 4, P>0.05

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Table 4 : Prevalence of S.haematobium in Relation to Sex in the study population

Sex	No Examined	No Infected	Prevalence (%)
Male	420	8	1.9
Female	180	4	2.2
Total	600	12	2

X^2 calculated = 0.06; X^2 tabulated = 3.841, df= 1, P>0.05

Table 5 : Prevalence of the parasite in relation to different occupational groups in the senatorial zone.

Occupation	No Examined	No Infected	Prevalence (%)
Students	268	8	3.0
Civil servants	130	0	0
Farmers	124	3	2.4
Other	78	1	1.3
Total	600	12	2

X^2 calculated = 13.38; X^2 tabulated = 7.815, df= 3, P>0.05

Table 5 show the infection rate of the parasite according to different occupational group. The occupational groups are namely; students, farmers, civil servants and others. Students had the highest prevalence rate of 8(3.0%) followed by farmers 3(2.4%), others 1(1.3%) and no civil servant were infected with parasite during the study. The prevalence rate of the parasite in the different occupational groups was statistically significant (P<0.05). However, prevalence rate among the available water sources in the area are pipe-borne water, Borehole, pool/pond, River/Stream and drawn well. Individuals whose sources of water supply for domestic use were streams recorded highest prevalence 25(9%) followed by pool/pond 5(5%) and pipe bond water had the least 1(0.8%) while no infection in borehole and drawn well. The difference in the infection rate in different occupational group in the study area was statistically significant (P<0.05) as show in table 6.

Table 7 shows the Infection rates in an individual who's used different types of toilet facilities. The available toilet facilities are namely water closet toilet, bucket, pit latrine, bush and those who use anywhere to defecate. Individuals who use bush to defecate recorded highest infection rate of 8 (8%) followed anywhere, pit latrine and water closet system with 1(2.5%), 2(0.7%) and

Table 6 : prevalence of the parasite among inhabitant with different source of water.

Source of Water	No Examined	No Infected	Prevalence (%)
Pipe borne	130	1	0.8
Pool/pond	40	2	5
Borehole	250	0	0
River/stream	36	9	25
Drawn well	144	0	0
Total	600	12	2

X^2 calculated = 163.07; X^2 tabulated = 7.815, df= 4, P>0.05

1(0.6%) respectively. During the study no individual found using buckets as toilet facility. The prevalence rate among the study population using different type of toilet facilities is significantly different (p<0.05).

Table 7 : Prevalence of the parasites among different users of toilet facilities during the study.

Toilet facility	No Examined	No Infected	Prevalence (%)
W.C Toilet	160	1	0.6
Bucket	0	0	0
Pit latrine	300	2	0.7
Bush	100	8	8
Anywhere	40	1	2.5
Total	600	12	2

X^2 calculated = 11.03; X^2 tabulated = 7.815, df= 4, P>0.05

Table 8 shows relationship between numbers of snails that shed cercariae and the average water quality values. The average pH, temperature and dissolved oxygen of the water samples collected were measure using appropriate meters and found to range from 7.0 to 8.5, 22.2 to 31.90C and 7.0 to 8.3mg/l respectively. Only one type of intermediate host of the parasite (snails) were encountered during the study in this zone (Bulinusglobosus). 422Bulinusglobosus was collected and examined and only 21(4.9%) were found infected or shed cercariae during the study. The month that cercarieawere shed had the lowest pH and temperature values but highest dissolved oxygen. Table 10 shows relationship between numbers of snails that shed cercariae and the average water quality values.

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Table 8 : Relationship between Snails Shedding *Cercariae* and average water quality values

Month	No of snails collected	No of snails Shed cercariae	Average Water Quality Values		
			pH	Temp $^{o}_{C}$	Dissolve oxygen mg/l
January	42	2	7.0	22.2	8.3
February	39	0	7.0	24.7	7.9
March	12	0	7.2	27.8	7.5
April	18	0	7.8	31.9	7.0
May	7	1	8.1	29.0	7.4
June	0	0	8.2	28.6	7.4
July	0	0	8.0	25.1	7.8
August	0	0	8.5	24.3	8.0
September	37	2	7.7	25.0	7.9
October	78	6	7.4	26.0	7.7
November	103	6	7.2	24.1	8.0
December	86	4	7.1	22.3	8.3
Total	422	21			

The entire Bauchi State experience two climatic seasons, the dry season (October to April) and the wet season (May to September). This study includes both the data in two seasons. The snail vectors species encountered during the study showed a great seasonal variation in all selected site with the peak at the beginning of dry season. This observation agrees with the earlier reports (23, 31). Out of 422 Bulinusglobosus snails examined only 21(4.9%) shed infective stage, (cercariae) of the parasite. This means the snail vectors were not harbouring the infective stage of the parasite in this zone. However, the infection in different location of the study area showed that Ganjuwa had the highest prevalence rate followed by Misau and Dambam local government had the least with 3.3%, 1.3% and 0.7% respectively. This pattern of infection in different locations within the same study area was similar to the report (11, 30). The major factors that might be responsible for these patterns are low literacy level, poor sanitation due to lack of basic amenities such as water, inadequate and indiscriminate disposal of human wastes, migration of infected individuals from endemic areas and high water contact activities such as irrigation activities, recreation and other related activities.

The overall prevalence of 12(2%) of the parasite (schistosomiasis) was observed and only Schistosoma haematobium egg were found in the study area. This indicates low endemicity of the parasite in the study area. These observations is in agreement with the earlier reports by (6,10)in Bauchi and Yobe states respectively which all are neighboring state to the study area. The low prevalence of the disease, recorded in this study area may probably be due to the fact that some of the localities are urban settlements with improved water supply and toilet facilities. However the snail vector are not harbouring the infective stage of the parasite as only 21(4.9%) of the snail vectors shed cercariae in their streams. The infection rate is higher in male than female counterpart, infection rate in individual with different occupational group with students and farmers had the highest prevalence rate than other occupational groups and also is higher in different age group with age 11-20 had the highest prevalence. The different between infection rate was not significant but individual with different occupational group and different age a group were all statistically significant ($p>0.05$). (34) report said the main groups at risk are school age children, specific occupational group (fishermen, irrigation workers, farmers) , woman and other groups using infected water for domestic purposes. (20) reported that infection in pre- school and school children was primarily due to exposure occasioned by washing, bathing, dry season farming, and fishing activities. Also the main reason of different between male and female would be due to the greater water contact

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activities by male compared to their female counterpart. Female mature early when compare to the male therefore, they restricted socially to water contact activities. These agree with (1, 5, 14, 32) in Niger, Kundiga,Danjarima and Bauchi respectively.

However, three physico-chemical parameters were measured for the purpose of this study i.e pH, temperature and dissolved oxygen. The average of these physico-chemical parameter values of the selected water bodies were taken and found were to be within the range that can support snail breeding. These values are in consonance with those recorded by other researchers such as (22, 23) in Imo and Bauchi states respectively. Low populations of snails were found in water bodies with low dissolved oxygen or even absent in some cases. This study has corroborate that dissolved oxygen in water bodies plays an important role in snail breeding, even if all other parameters are within the normal range as observed by (22). Only one species of snail were found and collected during the study 422 Bulinusglobosus and out of this only 21 (4.9%) where infected or shed cercariae.

In conclusion, as only 12(2%) out of 1200 samples (urine and stools 600 each) examined were positive with Schistosoma haematobium eggs and out of 422 snail vectors examined only 21(4.9%) were harbouring the infective stage of the parasite (cercariae). From the result of this study, it is concluded that the disease had low endemicity in the study area and is showing decline pattern when compare with previous studies. Therefore, proper health education to continue discourage people from urinating and defecating in or near open water as well as periodic survey of the water bodies in the area for snail intermediate hosts control is recommended for eradication of the disease and avoidance of further separation of the parasite in the study area.

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Anti-Osteoporotic Effects of Alendronate and Sitagliptin in Streptozotocin Induced Type 2 Diabetes Mellitus in Ovariectomized Rats

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Abstract

Diabetes mellitus is a metabolic disorder identifies as hyperglycaemia and osteoporosis is a bone disorder within which quality of bone and bone mineral density decline. Diabetes in osteoporotic patients is remarkably increased risk of bone fracture. Alendronate, a bisphosphonate first line therapy for osteoporosis treatment which prevents bone fracture by inhibiting osteoclast. Sitagliptin, an oral antidiabetic agent used for the treatment of type II diabetes mellitus by inhibiting Dipeptidyl peptidase-4 activity. Sitagliptin may regulate bone homeostasis by inhibiting osteoclast & supressing osteoclast differentiation. The present study was to investigate the anti-osteoporotic effect of sitagliptin and alendronate on bone mechanical properties in streptozotocin (STZ)induced diabetes in overiectamized rats (OVX). 30 female Wistar rats weighing from 180-250 g were divided into five groups each of 6 rats. Osteoporosis was induced by bilateral ovariectomy.After seven days of surgery the type 2 diabetes mellitus was induced by single intraperitoneal injection of streptozotocin (50 mg/kg) and nicotinamide (110 mg/kg). Groups III, IV and V were treated with alendronate (3 mg/kg), sitagliptin (30 mg/kg) and combination respectively for 42 days. The body weight of sitagliptin (30 mg/kg) and concurrent administration of alendronate (3 mg/kg) and sitagliptin showed significant increased compared to OVX-STZ groups. There is improvement in serum calcium and ALP in sitagliptin, alendronate and combination groups compared to OVX-STZ groups. Alendronate and combination groups showed significant increase in bone weight compared to STZ-OVX group. There is no significant changed in bone length and diameter. The bone mineral mass and three point bending test significantly increased in alendronate and combination groups compared to OVX-STZ groups. The concurrent administration of alendronate and sitagliptin showed beneficial effects in STZ induced type 2 diabetes mellitus in OVX rats.

Key words : Ovariectomy, Diabetes,Alendronate, Sitagliptin, Serum Calcium.

1. Introduction

Osteoporosis is a progressive chronic disease characterized by decreased bone mass, bone quality, resulting in bone fragility related to an enhanced risk fracture(1). According to World Health Organization osteoporosis as a decrease in bone mass (50%) and bony quality (50%). Bone loss happens once the cellular events of bone formation are quantitatively larger [2]. A highest bone mass at skeletal maturity is taken into account to be the most effective protection against age related bone loss and consequent fracture risk. Calcium is in every of the foremost vital determinants and nutrient factors to determine the peak bone mass in young adults. There usually aren't any symptoms within initial stages of bone loss. However once bones are weakened by osteoporosis, signs and symptoms that include back pain caused by a fractured or collapsed vertebra, loss of height over time, bone fracture that occurs much more easily than expected (3).

Diabetes mellitus has direct and indirect deleterious effects on osteoblast function and bone formation. Clinical evidences showed that bone mineral density (BMD) was below normal range in patients with type 1 diabetes mellitus, a subtype of diabetes caused by inability of pancreatic beta cells to secrete insulin (4). Diabetes associated decrease in BMD and weakened bone structure, of difficult pathophysiology, is taken in to account to be one of the major factors that cause bone fragility and elevated incidence of fractures in diabetic patients. Hyperglycemia can affect the bone density through different mechanisms.Toxic effects caused by high levels of glucose could directly decrease the osteoblast function and number.High levels of glucose might independently alter the amount of osteoblast gene expression through

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the osmotic and non-osmotic pathways. These changes lead to inhibition of bone forming cell maturation and bone mineralization.Impairment of osteoblast maturation, caused by high glucose levels, lead to an impaired response to 1, 25 hydroxy vitamin D3. This indirectly causes the down regulation of vitamin D receptors (5).High glucose levels, through non-enzymatic pathways, might induce glycationof numerous proteins and manufacture the products called advanced glycosylation end-products (AGEs). These products are seen in numerous tissues of diabetic subjects and are presupposed to be concerned in pathogenesis of diabetes. It appears hyperglycemia and AGEs have a serious role in fragility of bones in both type of diabetes. In cortical bone, accumulation of AGEs causes a rise in production of cross-links between collagens. Though this method can enhance the rigidity and hardness of collagen, it doesn't have an effect on the bone mineralization. Indeed there's a negative relation between AGEs and size and fragility of the human trabecular bone that might justify the raised bone fragility and fracture in diabetic subjects. Moreover, apart from the direct effects of high glucose, accumulation of AGEs encompasses a direct inhibitory effect on the proliferation and differentiation of bone cells (6). Production and accumulation of AGEs will induce the cellular cell death through production of reactive oxygen species (ROS) and oxidative stress(7).

Alendronate is a nitrogen containing bisphosphonates that's used for the treatment of some type of osteoporosis. It's taken orally and is usually recommended along with vitamin D, calcium supplementation, and lifestyle changes. The mechanism of nitrogen-containing bisphosphonates (eg,pamidronate, alendronate, risedronate, ibandronate, and zoledronate) inhibit a key enzyme, farnesyl pyrophosphate synthase, within the mevalonate pathway, thereby preventing the synthesis of isoprenoid compounds that are important for the posttranslational modification of little guanosine triphosphate (GTP)-binding proteins (which are GTPases) like Rab, Rho, and Rac. The inhibition of protein prenylation and also the disruption of the function of those key regulatory proteins describe the loss of osteoclast activity(8).

Sitagliptin is an oral anti-diabetic agent is often used as mono-therapy or in combined therapy with different oral antidiabetic drugs within the treatment of T2DM by inhibiting Dipeptidyl peptidase-4 activity. Sitagliptin would decrease bone loss and increase bone strength in diabetic rats by decreasing bone resorption independent of glycemic management (9). Hence from the above background, the present study was to investigate the anti-osteoporotic effects of alendronate and sitagliptin in STZ inducedtype 2diabetesin OVX rats.

2. Materials and Methods

Animals

30 female Wistar rats weighing from 180-250 g were housed in plastic cages at maximum of 3 per cage. The animal was obtained from Central Animal House, JSS College of Pharmacy, Ootyand were maintained under controlled environmental conditionson alternate 12 hours dark/light, temperature 21 ± 2°C. commercial pelleted feed and water ad libitumwas provided to animals. All the experiments were performed after obtaining prior approval fromCPCSEA and IAEC (Approval No.: JSSCP/OT/MPharm/10/2018-2019. The rats were acclimatizedto the experiment conditions for 5 days before initiating the experimental procedure.

Chemicals and reagents

Streptozotocin (STZ) was purchased from HI-Media, India. Alendronate and sitagliptin were purchased from Cipla and Sun Pharma respectively. Glucometer (Accu-Chek) was purchased from (Roche Diabetes Care, India),ALP and calcium kits were purchased from Q-Line Diagnostic Systems and Ensure Biotec. Pvt. Limited, India. All other chemicals and reagents were analytical grade.

Induction of osteoporosis

Osteoporosis induction in animal was by bilateral ovariectomy which involved the removal ofboth the ovaries and type 2 diabetes mellitus was induced by STZ and nicotinamideadministration.

Surgery procedure

The animal was anaesthetized by 50 mg /kg ketamine and 40 mg/kg xylazine injectedthrough intra-peritoneal route. The anesthetized animal was shaved on the ventral region below the ribs.The position of the ovaries was located by feeling the fat pads surrounding it with thethumb.Incision was made in the middle of the rat using No. 23 scalpel blade.A small incision was made in the muscle layer using a scalpel blade, fine scissors wasused to make incision.The ovary surrounded by a variable amount of fat was pulled out of the incision.A ligature was placed in the fallopian tube on both sides to control bleeding. The ovarieswere removed above the ligature at the junction of the oviduct.The abdomen was properly closed by proper ligation and suturing(10).

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Induction of type 2 diabetes mellitus

The rats were induced diabetic after two weeks of ovariectomy.Single dose of STZ 50 mg/kg was injected intraperitoneally after 15 min followed by intraperitonealadministration of nicotinamide (110 mg/ kg).The serum glucose level was measured after 72 h and serum glucose level more than 250 mg/dl were considered as diabetic rats (11).

Experimental design

After successful induction of osteoporosis along with type 2 diabetes mellitus rats were divided into 5 groups of 6 rats per group.

Group I : Sham
Group II : OVX-STZ
Group III : OVX-STZ+Alendronate (A) (3mg/kg p.o)
Group IV : OVX-STZ+Sitagliptin(S)(30 mg/kg p.o)
Group V : OVX-STZ+Alendronate(3mg/kg p.o)+ Sitagliptin (30 mg/kg p.o)

Group I served as sham and group II served as an OVX-STZ. Group III, IV and V were treated with Alendronate (3 mg/kg), Sitagliptin (30 mg/kg) and Alendronate (3mg/kg p.o) + Sitagliptin (30 mg/kg p.o)respectively for 42 days. The body weights and serum glucose level were measure at every 14 days. The blood was collected at the end of treatment for serum alkaline phosphatase (ALP) and calcium estimation. At the end of the treatment animals were sacrificed and bones (tibia & femur) were collected and stored in 10% formalin.

Biochemical parameters

Rats were monitored for every 14 days regarding body weight and serum glucose. On day 42, the rats were fasted for 12 hours and anaesthetized using (50 mg/kg ketamine plus 5 mg/kg diazepam, intraperitoneally), approximately 3 mL of blood was collected by cardiac puncture, centrifuged at 4000 rpm for 10 min for separation of serum. Each serum sample was stored in a clean sterile micro centrifuge tubes at -800 C until analysis.

Analytical measurements (12, 13)

The serum levels of glucose was measured using Accu-chek (Roche Diabetes Care, India), by tail vein puncture, calcium and alkaline phosphatase (ALP) were assayed for each rat with specific enzyme kits, [Ensure Biotech Pvt limited and Q Line Diagnostic systems, India] that were used according to the manufacturer's instructions

Bone length, diameter and mass (14)

Right femur length and diameter was calculated using digital vernier caliper. Right tibia and femur bones was cleaned from the surrounding tissues and stored in 4% formaldehyde. Bone mass was measured using digital balance.

Bone mineral mass (15)

Initial right femur bone weight was taken using balance. Bone was placed in crucible dish and ashed at the temperature of 600° C for 48hours in the incinerator. Ash was collected and weighed. The ratio of the mass of bone to the bone mass was determined.

Three point bone bending test

Bone was placed in the two triangle which serves as base 2 point and another point is present perpendicular to it. Constant force was applied to bone by rotating the screw present at top. Bone breaking time was noted for each group.

Statistical analysis

The data are represented as mean ± SEM. Body weight and serum glucose of animals were analysed by two-way ANOVA and biochemical except serum glucose, mechanical property wasanalysed by one-way ANOVA followed by Bonferroni multiple post hoc test p values ($p \leq 0.05$) was considered notably. The analysis was carried using GraphPad prism 6 software.

3. Results and Discussion

***Effects of alendronate and sitagliptin on body weight* :**

The results of body weight in OVX-STZ rats are given in (Figure 1). The result showed that body weight

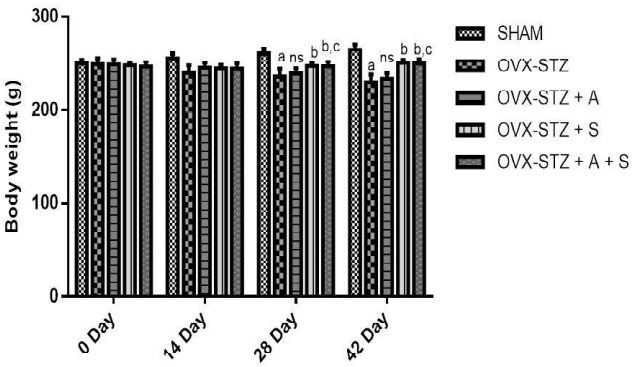

Figure 1 : Effects of alendronate and sitagliptin on body weight
The results were expressed as mean ± SEM.; n=6,
[a] significantly different vs Sham ($p<0.05$),
[b] significantly different vs OVX-STZ ($p<0.05$), csignificantly different vs alendronate ($p<0.05$),
[ns] non-significant compared to OVX-STZ group
Two-way ANOVA followed by Bonferroni multiple comparison post-test

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significantly ($p < 0.05$) decrease in OVX-STZ group when compared to Sham group. On 42 days chronic treatment with sitagliptin and concurrent administration of alendronate and sitagliptin showed significant increase ($p < 0.05$) in body weight compared to OVX-STZ group however alendronate showed no significant change in body weight compared to OVX-STZ rats. Concurrent administration of alendronate and sitagliptin showed no significant changed in body weight when compared to sitagliptin treated groups.

Effects of alendronate and sitagliptinon serum glucose :

The results of serum glucose in OVX-STZ rats are given in (Figure 2). The result showed that serum glucose significantly ($p< 0.05$) increase in OVX-STZ group when compared to sham group. On 42 days chronic treatment with sitagliptin and concurrent administration of alendronate and sitagliptin, showed significant ($p<0.05$) decrease in serum glucose level when compared to OVX-STZ group however alendronate did not show any change in serum glucose as compared to OVX-STZ group. Concurrent administration of alendronate and sitagliptin showed significant ($p<0.05$) decrease in serum glucose level compared to alendronate group.

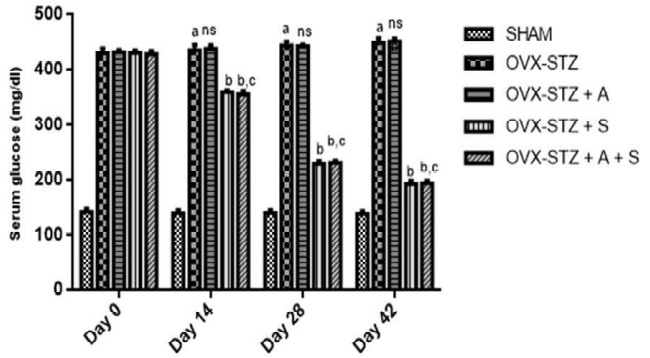

Figure 2 : Effects of alendronate and sitagliptin on serum glucose

The results were expressed as mean ± SEM.; n=6,
[a] significantly different vs Sham ($p<0.05$),
[b] significantly different vs OVX-STZ ($p<0.05$),
[c] significantly different vs alendronate ($p<0.05$),
[ns] non-significant compared to OVX-STZ group
Two-way ANOVA followed by Bonferroni multiple comparison post-test

Effects of alendronate and sitagliptin on serum calcium :

The results of serum calcium in OVX-STZ rats are given in (Figure 3). The result showed that serum calcium significantly ($p<0.05$) increase in OVX-STZ group when compared to Sham group. On 42 days chronic treatment

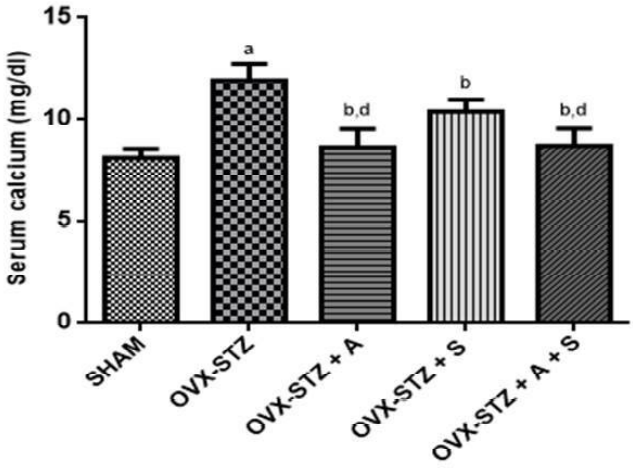

Figure 3 : Effects of alendronate and sitagliptin on serum calcium

The results were expressed as mean ± SEM.; n=6,
[a] significantly different vs sham ($p<0.05$),
[b] significantly different vs OVX-STZ ($p<0.05$),
[d] significantly different vssitagliptin ($p<0.05$)
One-way ANOVA followed by Bonferroni multiple comparison post-test

with alendronate, sitagliptin and concurrent administration of alendronate and sitagliptin groups showed significant ($p<0.05$) decrease in serum calcium when compared to OVX-STZ group. Concurrent administration of both drugs showed significant ($p<0.05$) decrease in serum calcium compared to alendronate and sitagliptin individual treated groups.

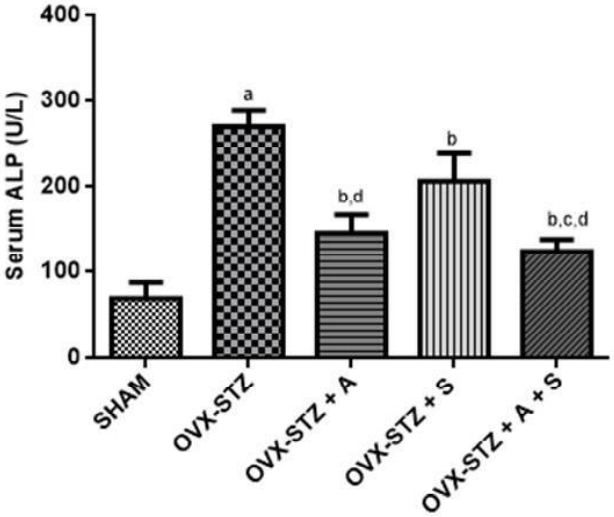

Figure 4 : Effects of alendronate and sitagliptin on serum alkaline phosphatase

The results were expressed as mean ± SEM.; n=6,
[a] significantly different vs sham ($p<0.05$),
[b] significantly different vs OVX-STZ ($p<0.05$),
[c] significantly different vs alendronate ($p<0.05$),
[d] significantly different vssitagliptin ($p<0.05$)
One-way ANOVA followed by Bonferroni multiple comparison post-test

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Effects of alendronate and sitagliptin on serum alkaline phosphatase :

The results of serum alkaline phosphatase in OVX-STZ rats are given in (Figure 4). The result showed that serum ALP has been significant ($p<0.05$) increase in OVX-STZ group when compared to Sham group. On 42 days chronic treatment with alendronate, sitagliptin and combination of both drugs showed significant ($p<0.05$) decrease when compared to OVX-STZ group. Concurrent administration of alendronate and sitagliptin showed significant ($p<0.05$) decrease in serum ALP compared to alendronate and sitagliptin individual treated groups however alendronate group show significant decrease in serum ALP compared to sitagliptin group.

Effects of alendronate and sitagliptin on bone weight :

The results of bone weight in OVX-STZ rats are given in (Figure 5 and 6). The result showed that bone weightsignificantly ($p<0.05$) decrease in OVX-STZ group when compared to sham group. Alendronate and combination of both drugs showed significant ($p<0.05$) increase in bone weight when compared to OVX-STZ group however. Combination of both drugs showed significant ($p<0.05$) increase in bone weight compared to sitagliptin however no significantdifference when compared to alendronate.

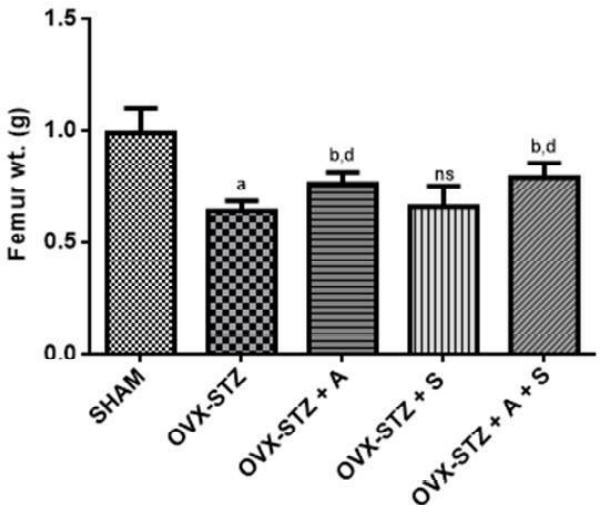

Figure 5 : Effects of alendronate and sitagliptin on femur weight
The results were expressed as mean ± SEM.; n=6,
[a] significantly different vs sham ($p<0.05$),
[b] significantly different vs OVX-STZ ($p<0.05$),
[d] significantly different vssitagliptin($p<0.05$)
[ns] non-significant compared to OVX-STZ
One way ANOVA followed by Bonferroni multiple comparison post-test

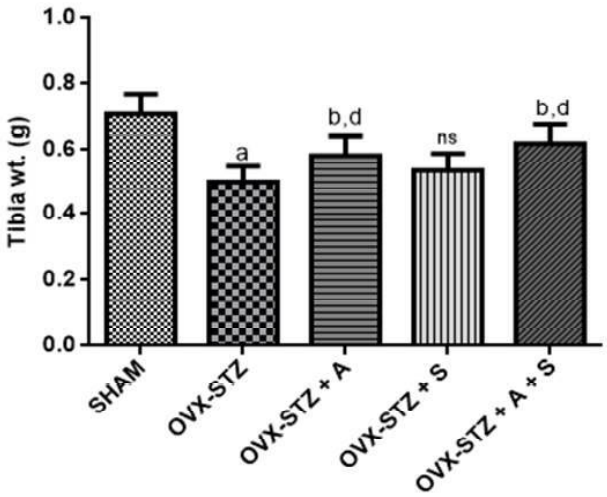

Figure 6: Effects of alendronate and sitagliptin on tibia weight
The results were expressed as mean ± SEM.; n=6,
[a] significantly different vs sham ($p<0.05$),
[b] significantly different vs OVX-STZ ($p<0.05$),
[d] significantly different vssitagliptin ($p<0.05$)
[ns] non-significant compared to OVX-STZ
One-way ANOVA followed by Bonferroni multiple comparison post-test

Effects of alendronate and sitagliptinon bone length, diameter and mass :

The results of bone length and diameter in OVX-STZ rats are given in (Table 1). The result showed that there is no significant difference in bone length and diameter

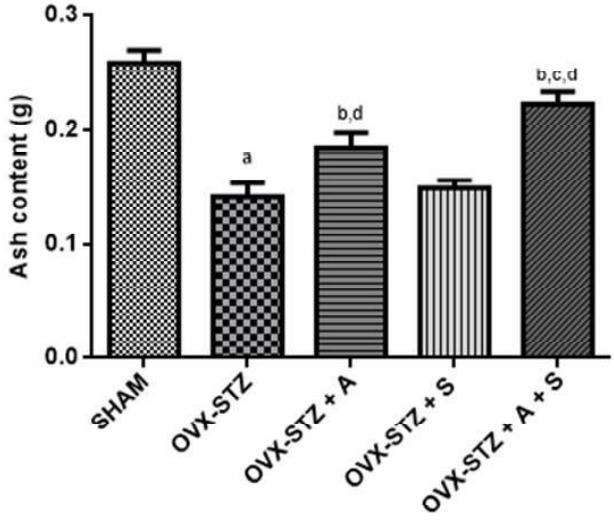

Figure 7 : Effect of alendronate and sitagliptinon bone mineral mass
The results were expressed as mean ± SEM.; n=6,
[a] significantly different vs sham ($p<0.05$),
[b] significantly different vs OVX-STZ ($p<0.05$),
[c] significantly different vs alendronate ($p<0.05$),
[d] significantly different vssitagliptin ($p<0.05$)
One-way ANOVA followed by Bonferroni multiple comparison post-test

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Table 1: Effects of alendronate and sitagliptin on bone length and diameter

Groups	Length		Diameter
	Femur length (mm)	Tibia length (mm)	Femur (mm)
SHAM	32.90 ± 0.485	35.32 ± 1.034	2.44 ± 0.092
OVX – STZ	32.31 ± 0.849^{ns}	34.53 ± 1.149^{ns}	2.34 ± 0.078^{ns}
OVX-STZ + A	33.24 ± 0.577^{ns}	35.16 ± 0.926^{ns}	2.34 ± 0.094^{ns}
OVX-STZ + S	32.98 ± 0.519^{ns}	34.20 ± 1.119^{ns}	2.36 ± 0.078^{ns}
OVX-STZ + A + S	32.55 ± 0.526^{ns}	34.72 ± 0.992^{ns}	2.32 ± 0.103^{ns}

Data expressed as mean ± SEM.; n=6.
^{ns} - non significant compared to all groups
One-way ANOVA followed by Bonferroni multiple comparison post-test

in OVX-STZ group when compared to sham group and treatment groups.

The results of bone mineral mass in OVX-STZ rats are given in (Figure 7). The result showed that there is significant ($p<0.05$) decrease in bone mineral mass in OVX-STZ group when compared to sham group. Alendronate and combination of both drugs showed significant ($p<0.05$) increase in bone mineral mass compared to OVX-STZ group however sitagliptin did not showed significant difference compared to OVX-STZ group. Combination group showed significant ($p<0.05$) increase in bone mineral when compared to alendronate and sitagliptin individual treated groups.

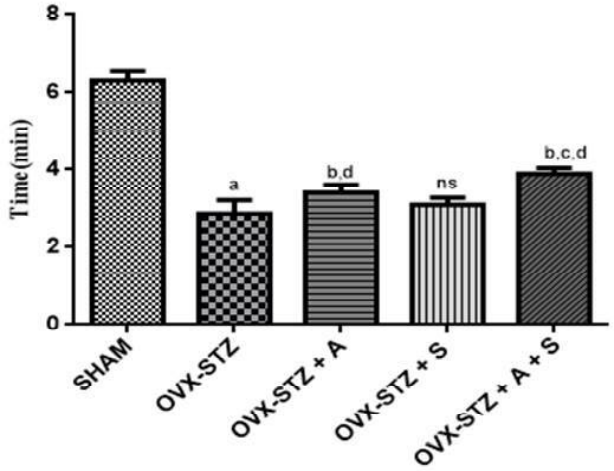

Figure 8 : Effect of alendronate and sitagliptin on three point bending test
The results were expressed as mean ± SEM.; n=6,
[a] significantly different vs sham ($p<0.05$),
[b] significantly different vs OVX-STZ ($p<0.05$),
[c] significantly different vs alendronate ($p<0.05$),
[d] significantly different vssitagliptin ($p<0.05$),
[ns]-non significant compared to OVX-STZ
One-way ANOVA followed by Bonferroni multiple comparison post-test

Effects of alendronate and sitagliptin on three point bending test :

The results of three-point bending test in OVX-STZ are given in (Figure 8).The result showed that there is significant ($p<0.05$) decrease in time in OVX-STZ group when compared to sham group. Alendronate and concurrent administration of both drugs showed significant ($p<0.05$) increase time compared to OVX-STZ group however sitagliptin showed no significant difference in time compared to OVX-STZ group. Concurrent administration of both drugs showed significant ($p<0.05$) increase in time compared to alendronate and sitagliptin individual treated groups.

3. Results and Discussion

Effects of insulin insufficiency on the bone could also cause bone and mineral deformity in diabetic patient. Each type 1 and type 2 diabetes mellitus have been related to bone diseases such as osteoporosis. Osteoporosis, a metabolic bone disorder, is becoming more and more prevailing in older population. Osteoporosis occurs due to an inequality between osteoblast and osteoclast function and the diabetes induced osteoporosis is unclear (16, 17).The present study was performed to evaluate the anti-osteoporotic effect of alendronate and sitagliptin in STZ induced type 2 diabetes mellitus in ovariectomized rats.

Chan et al.,(18) reported that in diabetic osteoporosis condition, there is decrease in body weight in OVX-STZ group. This is due to insufficient insulin which stops the body to receiving glucose from the blood. When this occurs, the body starts utilizing fat and muscle for energy and reduced in body weight. The similar result were found in the present study where body weight of all OVX-STZ rats were decreased initially but after 42 days of treatment,

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there was gradual increase in body weight in sitagliptin and combination treated groups. Whereas, alendronate treated group decreased the body weight even after the treatment for 42 days. This indicates alendronate doesn't have any effect on diabetes in ovariectomized rats.

Results of the present study showed significant increase in blood glucose in OVX-STZ rats compared sham group. Farid et al. (19) were reported similar results. After 42 days of treatment, there was gradual decrease in blood glucose in sitagliptin and combination treated groups. Whereas, Alendronate treated group was not showing any change in blood glucose level as compared to OVX-STZ rats after the treatment for 42 days.

Calcium is one of the most vital minerals present in bone. It helps maintain bone strength, build and maintain bones. It was suggested that calcium absorption plays a significant role in bone turnover, and its deficiency results in a reduced bone mineralization (20). The present study results showed significant increase in serum calcium in OVX-STZ as compared to sham group and Hassan et al. (21)also reported similar results. The increase in serum calcium indicated that there is more bone resorption of in OVX-STZ. After 42 days treatment with alendronate, sitagliptin and combination of both drugs showed significant decreases in serum calcium compared to OVX-STZ rats.

ALP is an enzyme that is mainly found in liver and bone. Levels of this enzyme increases when bone cells are active. ALP helps in bone mineralization. Abnormal level of ALP in blood is due to bone disorder (22). Biochemical results revealed that serum ALP concentrations were increased in OVX-STZ group as compared to sham group. The same results were reported by Chan et al. (18). After 42 days treatment with Alendronate sitagliptin and combination of these drugs showed significant decreased in serum ALP level compared with the STZ-OVX group.

The change in the femur size & diameter and bones weight may be because of the bone mineral mass (23). The bone weights were significant increase in alendronate and combination treatment when compared to OVX-STZ induced rats. However,sitagliptin showed no significant change in bone weight compared to OVX-STZ rats. There was no significant change in bone length and diameter compared to sham group.

Three point bending test demonstrated the worsening of mechanical property of femur bone in OVX-STZ rats. The similar result reported by Y. Ko. et al. (23). Concurrent administration of alendronate and sitagliptin showed increased in activity when compared to OVX-STZ, alendronate and sitagliptin treated rats.

Diabetes in ovariectomized decreases the bone minerals which was detected in this study. The OVX-STZ group showed a decrease in the ash values (23). Alendronate and sitagliptin treatment increased ash values compared to OVX-STZ rats. It suggests that alendronate and sitagliptin have bone protective action. Concurrent administration of alendronate and sitagliptin showed increased in ash value when compared to OVX-STZ and alendronate treated rats.

4. Conclusion

The present study demonstrates a bone preserving effect of concurrent administration of alendronate and sitagliptin in OVX STZ induced type 2diabetes mellitus and this effect may be due to suppression of osteoclastogenesis. Our data gives apotential effect of combination of both drugs alendronate and sitagliptin by preventing osteoporosis of postmenopausal women with diabetes and further information need to be assessed in future preclinical and clinical studies.

Acknowledgements

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Conflict of Interest

The authors have no conflict of interest

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Perception and Satisfaction Among Single and Dual Users Malaysian Vapers Towards Electronic Cigarettes. A One Year Observational Study

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Abstract

In Malaysia and elsewhere in the world Electronic cigarette (EC) consumers, use the product either alone (single users, SUs) or in combination with conventional cigarettes (CCs) (dual users, DUs). However, the existing studies have limited to explore the long-term perceptions and satisfaction levels among SUs and DUs.The present study aimed to evaluate the perceptions and satisfaction levels among SUs and DUs after one year of EC use. A total of 218 SU and DUs participants were enrolled in the current study from the districts of Pekan and Kautan, state Pahang, Malaysia. The 70 were SUs, as verified by the carbon monoxide (CO) levels of <8 ppm, and the remaining were DUs, as verified by the CO levels of ?8 ppm. The participants were interviewed after 1 year of EC use regarding their perceptions and satisfaction levels to EC which were scored on a scale of 0-4, not sure to very sure. Overall, 33.3% of the participants were very much sure that ECs are an effective smoking cessation aid. However, 43.8 % of SU participants were very much sure that ECs are an effective smoking cessation aid then 30.2% of DUs. While perceptions of SUs regarding the safety of ECs was 78.9% as compared to 86.6% of DUs. After one year of EC use more SUs perceived positively satisfaction than DUs. However, there was no perception variance in both groups' users related to the safety of EC. Nevertheless, the EC understanding and its attainment are recommended to be confirmed further in diverse populations.

Key words : Electronic cigarette, conventional cigarette, perception, satisfaction, carbon monoxide

1. Introduction

Electronic cigarette (EC) is a relatively novel product, and its use as an electronic nicotine delivery system is spreading worldwide. EC is a battery-operated device available in various sizes and shapes, such as conventional cigarettes (CCs), pens and boxes. ECs do not release smoke; instead, they vaporises e-liquids (e-juice) that vapers inhale. Nicotine, propylene glycol (PG), glycerine and a couple of other ingredients mostly tobacco or fruit flavours are the chief constituents of e-liquids. The aim of developing ECs is to simply simulate the act of smoking via the nicotine contents of e-liquids while avoiding the noxious effects of tobacco smoke. Furthermore, ECs produce vapours that resemble smoke and are associated with most of the social and behavioural features of smoking like the hand to mouth action (1-3).

According to the Malaysian National Health and Morbidity Survey (NHMS, 2015), smokeless tobacco consumption, including EC use, in Malaysia increased from 0.7% to 10.9% during 2011-2015. Furthermore, the report speculated that the sudden increase in the consumption of smokeless tobacco products among Malaysian adults (?15 years of age) was due to the increased consumption of ECs (National Health and Morbidity Survey, 2015) (4). However, the 2016 Malaysian National E-cigarette Survey reported that the prevalence of EC users among Malaysian adults ?18 years of age was 3.2% (3.3% in urban and 2.9% rural areas), while the prevalence of combined EC use with tobacco cigarettes was 2.3% (5).

Furthermore, in a cross-sectional provincial survey among 429 Malaysian EC users, 85% of the participants believed that ECs are not as bothersome as CCs and can be used in public places (6). A survey by Factasia.org reported that more than three-quarters of the 400 participating Malaysian adult smokers believed that ECs are a promising substitute for CCs and were willing to use the product if it is legal, risk-free and as accessible as

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CCs (7). In Malaysia and elsewhere in the world, EC smokers use the product either alone (single users, SUs) or in combination with CCs (dual users, DUs). However, there are limited published data regarding the perceptions and satisfaction levels among Malaysian SUs and DUs after long-term EC use. Both SUs and DUs represent significant populations of the existing vaper community, and their opinions after longterm EC use may reveal perceived benefits and contrary effects related to EC usage. Therefore, the present study aimed to assess the perceptions and satisfaction levels among SUs and DUs after a year of EC use.

2. Materials and Methods

Study design

The prospective study was designed to assess the satisfaction and perceptions levels among current SUs and DUs in a natural setting over 1 year period.

Sample size

A total of 218 participants who consented to follow the study procedure were enrolled through convenience sampling. The participants were enrolled at a ratio of 2:1 (148 DUs and 70 SUs) as the majority of the EC users are DUs (4-5). Finally, data were collected from 82% (176) of the enrolled participants. Convenience sampling was implemented because random samples of SUs and DUs from the main study population were difficult to obtain. Thus, the above enrolment technique was the most feasible in terms of time and funding constraints.

Inclusion and exclusion criteria

The inclusion criteria were as follows: current single and dual-use of EC products since a minimum of 1 month, age of 18-65 years and good self-reported health. Exclusion criteria were as follows: use of any smoking cessation medicines, such as nicotine replacement therapy or varenicline, currently or within the previous year and dependency on any illegal drugs.

Study questionnaire

A pre-validated, English questionnaire was administered via interviews to collect data. The questionnaire was developed and pilot-tested among Malaysian SUs and DUs of ECs (8).

Settings and stratification

Study participants were selected from the semi-urban districts of Kuantan and Pekan in the state of Pahang, Malaysia. Smoking prevalence in the state of Pahang was 25.5% in the year 2015, which is more than the national smoking prevalence of 22.8% (4). The above study sites were selected due to time and capital constraints. Furthermore, these sites were more convenient in terms of accessibility to EC users.

Participants were stratified into two groups based on their smoking status: SUs [use of EC alone and measured carbon monoxide (CO) levels of <8 ppm] and DUs (use of both ECs and CCs and CO levels of $\geq$8 ppm) (Figure 1).

Data collection

Study data were collected between March 2015 and June 2016. Initially, each study participant was questioned independently for 25-30 minutes to collect sociodemographic details, smoking history (pack-years) and EC use history. More than 90% of the study participants understood and spoke English. For participants who did not know English, a translator (English to Bahasa Malay) was engaged. The same translator assisted in all interviews to avoid errors due to prejudice and inconsistencies.

At the final interview, each participant's perceptions and satisfaction levels towards ECs were reported on a scale of 0-4 (0 = not sure, 1 = less sure, 2 = moderately sure, 3 = very sure and 4 = very much sure). Participants' responses to queries related to whether ECs help in quitting smoking, reducing CC consumption, preventing relapse to smoking, controlling cravings for smoking and managing smoking-related withdrawal symptoms was assessed. Participants were assessed for satisfaction levels with the use of ECs as a smoking cessation aid. They were also asked whether they would recommend the use of ECs to their friends and kin as a smoking cessation aid. Besides, we documented any case of EC dependence and physical harms associated with EC use.

Ethical approval and consent to participate

The study questionnaire, protocols, consent forms, participant information sheet and study-related flyer to recruit the study subjects were approved by the Research Ethics Committee (IREC) of Kulliyyah of Medicine, International Islamic University Malaysia (IIUM), Kuantan on 9th October 2014, IREC no. 302 and by the National Medical Research Registration (NMRR.NO:15-180-24,825). Written consent was obtained from all the participants before their enrolment in the study

Data analysis

Categorical variables were summarised as frequencies and percentages, and continuous variables were

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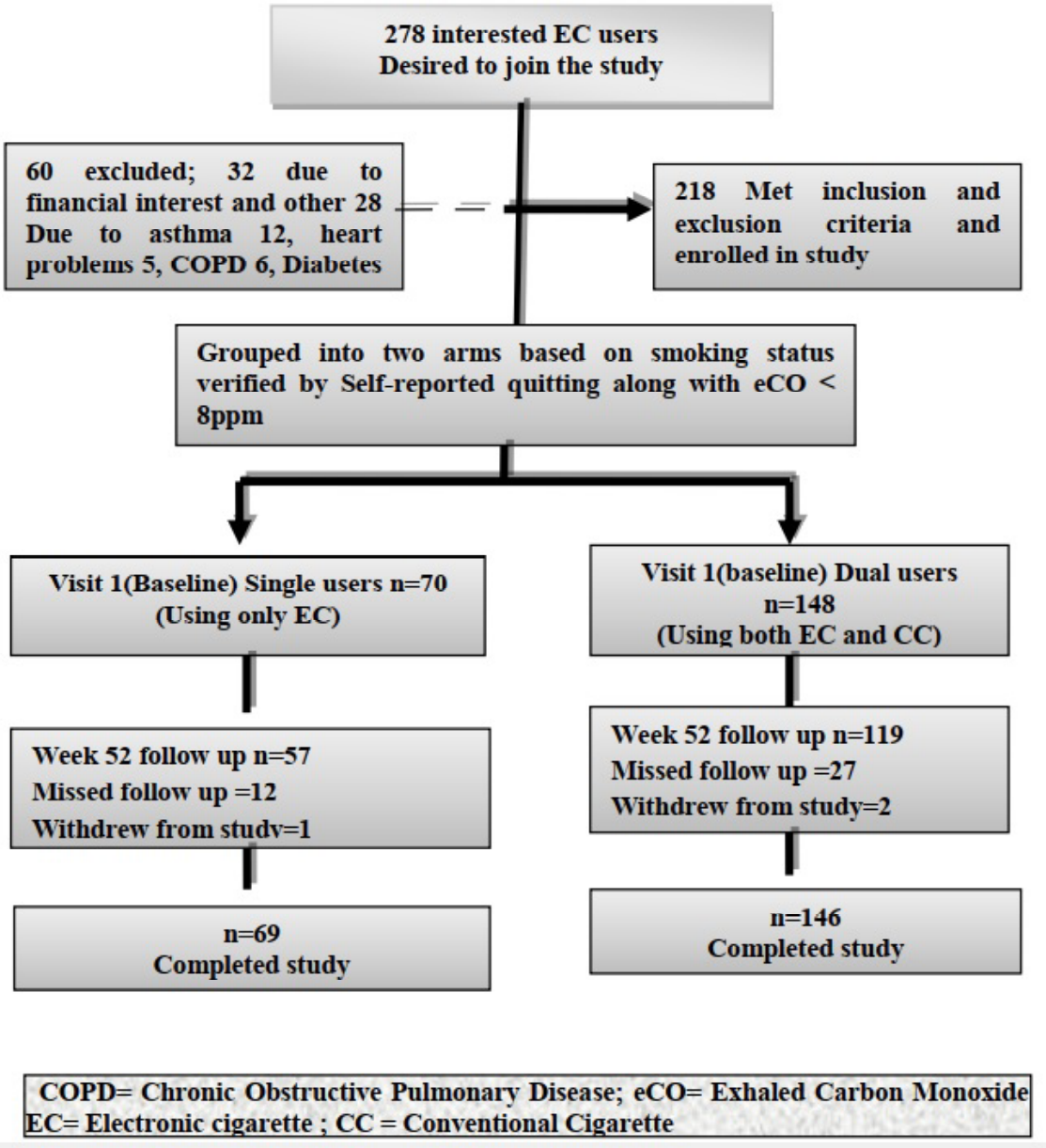

Figure 1 : Number of particpants at baseline and at week 52 among the single and dual users vapers

calculated as medians. Chi-squared test was applied for categorical variables, and independent t-tests were applied to compare mean differences between the groups. Mann-Whitney U test was used to compare nonparametric data between the groups. Statistical methods were two-tailed, and a p-value of <0.05 was considered significant. Intention to treat analysis was applied for study outcomes. Data of all participants who were interviewed at least once during the follow-up visits were included in the final analysis. Those who missed more than two interviews were excluded from the final analysis. Participants who were lost to follow-up were excluded (n = 3). Analyses were performed using the Statistical Package for Social Sciences (IBM®, SPSS® Inc., Chicago, IL) for Windows version 21.

3. Results and Discussion

Baseline characteristics of the participants

Demographic characteristics of both groups did not vary. In both, the groups, the median age was 23 years and nearly 98% of the participants were males. More DUs than SUs were unmarried at enrolment. Both the groups showed the same race distributions (p = 0.632). Majority of the study participants were Malays (80%), followed by Chinese (11.9%) and Indians (1.8%). Approximately 73% of the study participants were either studying or held a diploma or degree. The two groups did not differ in terms of profession and income. At the initial visit, physical and behavioural dependences on ECs were the same in both the groups (p = 0.668).

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Table 1 Perception and satisfaction levels among SUs and DUs after one year period of EC use

Perception and Satisfaction	Groups	Not sure, n (%)	Less sure, n (%)	Moderately sure, n (%)	Very sure, n (%)	Very much sure, n (%)	Median	P (2T)
1. Did e-cigarette help in quitting smoking?	DUAL USERS (n = 119) SINGLE USERS (n = 57) TOTAL USERS (n = 176)	46 (38.7) 8 (14) 54 (30.7)	27 (22.7) 9 (15.8) 36 (20.5)	11 (9.2) 5 (8.8) 16 (9.1)	30 (25.2) 26 (45.6) 56 (31.8)	5 (4.2) 9 (15.8) 14 (8)	1.00 3.00	<0.001
2. Did e-cigarette help in reducing tobacco smoking?	DUAL USERS (n = 119) SINGLE USERS (n = 57) TOTAL USERS (n = 176)	16 (13.4) 1 (1.8) 17 (9.7)	19 (16) 2 (3.5) 21 (11.9)	30 (25.2) 14 (24.6) 44 (25)	54 (45.4) 35 (61.4) 89 (50.6)	0 (0) 5 (8.8) 5 (2.8)	2.00 3.00	<0.001
3. Did e-cig help in controlling relapse from tobacco smoking?	DUAL USERS (n = 119) SINGLE USERS (n = 57) TOTAL USERS (n = 176)	28 (23.5) 0 (0) 28 (15.9)	24 (20.2) 12 (21.1) 26 (20.5)	30 (25.2) 10 (17.5) 40 (22.7)	36 (30.3) 35 (61.4) 71 (40.3)	1 (.8) 0 (0) 1 (0.6)	2.00 3.00	<0.001
4. Did e-cigarette help in controlling craving of smoking?	DUAL USERS (n = 119) SINGLE USERS (n = 57) TOTAL USERS (n = 176)	9 (7.6) 1 (1.8) 10 (5.7)	26 (21.8) 9 (15.8) 35 (19.9)	47 (39.5) 12 (21.1) 59 (33.5)	36 (30.3) 35 (61.4) 71 (40.3)	1 (0.8) 0 (0) 1 (0.6)	2.00 3.00	0.001
5. Did e-cigarette help to managed withdrawal symptoms of smoking?	DUAL USERS (n = 119) SINGLE USERS (n = 57) TOTAL USERS (n = 176)	9 (7.6) 0 (0) 9 (5.1)	24 (20.2) 18 (14) 32(18.2)	48 (40.3) 12 (21.1) 60 (34.1)	38 (31.9) 37 (64.9) 75 (42.6)	00 00 00	2.00 3.00	<0.001
6. Will you suggest e-cigarette to your loved ones who want to quit smoking with the help of e-cig?	DUAL USERS (n = 119) SINGLE USERS (n = 57) TOTAL USERS (n = 176)	8 (6.7) 1 (1.8) 9 (5.1)	39 (32.8) 8 (14) 47 (26.7)	35 (29.4) 15 (26.3) 50 (28.4)	37 (31.1) 30 (52.6) 67 (38.1)	0 (0) 3 (5.3) 3 (1.7)	2.00 3.00	<0.001
7. Your satisfaction towards e-cigarette as an effective quit smoking aid.	DUAL USERS (n = 119) SINGLE USERS (n = 57) TOTAL USERS (n = 176)	10 (8.4) 1 (1.8) 11 (6.3)	47 (39.5) 19 (33.3) 66 (37.5)	26 (21.8) 12 (21.1) 38 (21.6)	30 (25.2) 19 (33.3) 49 (27.8)	6 (5) 6 (10.5) 12 (6.8)	2.00 3.00	<0.001
8. Have you scared about the side effects of e-cigarette?	DUAL USERS (n = 119) SINGLE USERS (n = 57) TOTAL USERS (n = 176)	2 (1.7) 0 (0) 2 (1.1)	98 (82.4) 45 (78.9) 143 (81.3)	17 (14.3) 11 (19.3) 28 (15.9)	2 (1.7) 1 (1.8) 3 (1.7)	00 00 00	1.00 1.00	0.316
9. Have you scared about the contents of e-cigarette liquids?	DUAL USERS (n = 119) SINGLE USERS (n = 57) TOTAL USERS (n = 176)	1 (0.8) 0 (0) 1 (0.6)	103 (86.6) 45 (78.9) 148 (84.1)	14 (11.8) 12 (21.1) 26 (14.8)	1 (0.8) 0 (0) 1 (0.6)	00 00 00	1.00 1.00	0.131
10. Did you scare of becoming addicted to the e-cigarette?	DUAL USERS (n = 119) SINGLE USERS (n = 57) TOTAL Users (n = 176)	8 (6.7) 7 (12.3) 15 (8.5)	65 (54.6) 22 (38.6) 87 (49.4)	41 (34.5) 20 (35.1) 61 (34.7)	5 (4.2) 8 (14) 13 (7.4)	00 00 00	1.00 1.00	0.255
11. Did you like the taste of e-cigarette?	DUAL USERS (n = 119) SINGLE USERS (n = 57) TOTAL USERS (n = 176)	-- -- --	3 (2.7) 0 (0) 3 (1.7)	69 (58) 38 (66.7) 107 (60.8)	46 (38.7) 18 (31.6) 64 (36.4)	1 (0.8) 1 (1.8) 2 (1.1)	2.00 2.00	0.609
12. Did you like the smell of e-cigarette?	DUAL USERS (n = 119) SINGLE USERS (n = 57) TOTAL USERS (n = 176)	-- -- --	3 (2.7) 0 (0) 3 (1.7)	71 (59.7) 34 (59.4) 107 (59.7)	44 (37) 22 (38.6) 66 (37.5)	1 (0.8) 1 (1.8) 2 (1.1)	2.00 2.00	0.545
13. Did you like to have more nicotine in your e- cigarette?	DUAL USERS (n = 119) SINGLE USERS (n = 57) TOTAL USERS (n = 176)	6 (5) 7 (12.3) 13 (7.4)	24 (20.2) 18 (31.6) 42 (23.9)	34 (28.6) 18 (31.6) 52 (29.5)	55 (46.2) 14 (24.6) 69 (39.2)	00 00 00	2.00 2.00	0.002
14. Did you like to have more concentrated vapour in your e-cig?	DUAL USERS (n = 119) SINGLE USERS (n = 57) TOTAL USERS (n = 176)	-- -- --	2 (1.7) 1 (1.8) 3 (1.7)	79 (66.4) 32 (56.1) 111 (63.1)	38 (31.9) 23 (40.4) 61 (34.7)	0 (0) 1 (1.8) 1 (0.6)	2.00 2.00	0.162
15. Is it easier to draw puff on e-cigarette compared to tobacco cigarette?	DUAL USERS (n = 119) SINGLE USERS (n = 57) TOTAL USERS (n = 176)	-- -- --	17 (14.3) 3 (5.3) 20 (11.4)	81 (68.1) 44 (77.2) 125 (71)	21 (17.6) 10 (17.5) 31 (17.6)	00 00 00	2.00 2.00	0.323
16. Did you scare that, if you stop e-cigarette use, you will start smoking again?	DUAL USERS (n = 119) SINGLE USERS (n = 57) TOTAL USERS (n = 176)	5 (4.2) 7 (12.3) 12 (6.8)	36 (30.3) 27 (47.4) 63 (35.8)	67 (56.3) 17 (29.8) 84 (47.7)	11 (9.2) 6 (10.5) 17 (9.7)	00 00 00	2.00 1.00	0.006

Perception and satisfaction of the 176 final visit participants were measured on a scale of 0-4 scales (0 = not sure; 1 = less sure; 2 = moderately sure; 3 = very sure; 4= very much sure). The data are nonparametric and expressed as median two-tailed p-value are calculated by the Mann-Whitney U test.

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Perception and satisfaction of EC use

The measured perceptions and satisfaction levels among both the groups are summarised in Table 1. All study participants were asked whether ECs helped in efforts to quit smoking. Overall, 31.89% of the participants were very sure that ECs helped them quit smoking. However, more SUs (median = 3) than DUs (median = 1) answered positively to this query ($p < 0.05$). Similarly, all participants were asked whether ECs helped reduce CC consumption. More than 50.6% of the participants were sure that ECs helped them reduce CC consumption. Again, more SUs (median = 3) than DUs (median = 2) answered positively ($p < 0.05$).

Furthermore, the participants were asked whether ECs contributed to preventing relapse to smoking. Nearly 40.3% of the participants were very sure that ECs helped to prevent relapse to smoking. In addition, all participants were asked about their level of satisfaction towards ECs as an effective smoking cessation aid. A total of 43.8 % of SU participants were very much sure that ECs are an effective smoking cessation aid then 30.2% of DUs. While perceptions of SUs regarding the safety of ECs was 78.9% as compared to 86.6% of DUs respectively. The views of both group users' related to other queries are reported in table 1.

The present study is the first of its kind in Malaysia. This study revealed the perceptions and satisfaction levels among SUs and DUs after 1 year of EC use. More than one-third of the participants believed that ECs helped them quit smoking, reduce CC consumption and prevent relapse to smoking. Furthermore, nearly the same proportions of SUs and DUs expressed that ECs helped them cope with the urge to smoke and related withdrawal symptoms. Besides, nearly one-third of the study participants were satisfied with EC use and rated it as an effective smoking cessation aid. However, to the queries regarding the effectiveness of ECs in helping to quit smoking and reduce CC consumption, more SUs than DUs responded positively.

The findings of the present study are consistent with those of previous studies reporting that vapers were satisfied with EC use and rated it as an effective smoking cessation aid (9-11). However, we also found that the EC users differed in their perceptions of the effectiveness of ECs as a smoking cessation aid. The results indicated that DUs were less satisfied with the effectiveness of ECs even after 1 year of EC use, which warrants further research. More than three-quarters of the study participants reported that they were less afraid of the side effects associated with EC and e-liquids. Currently, although the safety of ECs remains controversial, the majority of the study participants strongly expressed less apprehension regarding the harms of ECs. This perception of the safety of ECs is consistent with reports of previous studies in which participants believed ECs to be less harmful and less destructive than CCs (9-13). In terms of perceptions regarding addiction to ECs, nearly two-thirds of the participants were moderately afraid of becoming EC dependent. This result is also comparable with the results of previous studies in which vapers felt less dependent on ECs than on CCs (14). Experts on tobacco use believe that dependency on CC among smokers is due to not only nicotine but also some other chemicals in tobacco smoke, which reinforce the nicotine effects and lead to severe addiction to CCs (1,3).

Additionally, a severe addiction to CCs is due to the fast delivery of nicotine to the brain via pulmonary absorption, providing immediate satisfaction to smokers. During vaping, the nicotine supplied by ECs is poorly absorbed in the lungs of vapers. Consequently, less nicotine reaches the brain, leading to less satisfaction among users. However, the technology of ECs is advancing each day, and novel EC models like JUUL® deliver higher doses of nicotine than CCs, thus offering more satisfaction to vapers (15-16).

More than three-quarters of the participants preferred the taste and smell of ECs over those of CCs. However, three-quarters of the participants also expressed that ECs should produce thick vapours. Moreover, these participants stated that it was easier to draw puffs from ECs than from CCs. Similarly, previous studies have confirmed that majority of the users preferred the smell and taste of ECs but desired higher vapour production to make vaping as enjoyable as smoking (10-11). However, previously published studies have expressed concerns regarding these fascinating characteristics of EC because they may appeal to adolescents and non-smokers and encourage them to start vaping, leading to the development of nicotine addiction (17-19).

More than two-thirds of the participants felt that ECs should supply more nicotine to gain higher smoking satisfaction. The insufficient delivery of nicotine may be the reason most users craved for CCs. Generally, the nicotine supplied by ECs depends on nicotine concentration and propylene glycol proportion in e-liquids, puffing technique and device. Although the majority of the participants used third-generation EC devices, they used very low nicotine concentration (6 mg/

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ml) (20) which may be one of the reasons for participants' desire for a higher concentration of nicotine in ECs.

In addition, low nicotine supply from ECs may be due to the improper selection of nicotine base in e-liquids. The major ingredients of e-liquids include propylene glycol (PG) and vegetable glycerin (VG). E-liquids that have a 50 PG :50 VG formulation ratio deliver more nicotine than those that contain a 25 PG:75 VG formulation ratio. High supply of nicotine from e-liquids with 50 PG: 50 VG ratio is thought to be due to low boiling point of PG (187.6°C). Thus, e-liquids with a higher proportion of PG evaporate faster than those with a higher proportion of 25 PG:75 VG ratio where the boiling point of VG is 290°C. Therefore, e-liquids with high PG content deliver immediate nicotine supply and increase smoking satisfaction in users than base e-liquids with high VG content (21).

Regarding technical problems and physical injuries associated with EC use, less than one-tenth of the users reported technical problems with EC use. These problems included e-liquid leakage, device heating and rapid battery discharge. These results are consistent with those reported by Etter & Bullen (2011) and Farsalinos et al. (2014). However, technological problems can be rectified by elevating product standards and by developing new, innovative EC models.

No physical harm associated with EC use was reported by any participant. However, one case of e-liquid spilling with no injury or side effects was reported. Previous studies have reported accidental and intentional nicotine poisoning cases among children and adults (22-23). For the safety of vapers and the public, these problems can be solved by refilling e-liquids in childproof packaging and by standardising the bottle size for the maximum nicotine concentration of 20 mg/ml in ? 10 ml.

The present study has some limitations. The participants were mostly middle-aged Malay males with fewer Chinese and Indians from the Kuantan and Pekan districts. Additionally, only two regions in Malaysia were sampled due to constraints of time and funds. However, the baseline characteristics and EC use patterns of the study participants did not significantly differ from those of EC users in other regions of Malaysia. Additionally, demographic characteristics of the study participants were comparable to those of the participants of two national surveys, namely NHMS, 2015 (4) and NECS, 2016 (5).

The study participants spoke English and were in good health at the time of recruitment. Therefore, the outcomes of the present study may not be generalisable to the other populations with low educational qualifications and poor health status. Furthermore, the study participants comprised those who were already striving to quit smoking, which further limits any generalisation of the results. Of note, the present study revealed the perceptions of two types of EC users, particularly in terms of benefits and undesirable effects of ECs, which have hardly ever been reported before in Malaysia. Finally, the results of the present study indicate that SUs had a more positive opinion of EC than DUs after 1 year of EC use.

4. Conclusion

Both SUs and DUs generally perceived ECs well and were satisfied after 1 year of EC use. However, SUs were more optimistic and more satisfied with EC than DUs. Nevertheless, these study results warrant further validation in different populations.

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Availability of Data and Materials : The datasets generated and analysed during the current study are available from the corresponding author on reasonable request.

Competing interests : The authors declare that they have no competing interests.

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Investigation of Process Variables in the Development of Nateglinide Nanocrystals

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Abstract

Nateglinide (NTG) classified under the biopharmaceutical classification system (BCS) class II, an oral antidiabetic drug. Solubility is one of the main factors to improve its bioavailability. The present research developed Nateglinide nanocrystals to overcome its low solubility and high permeability by ultrasonic probe method and characterized by coulter counter analysis, zeta sizer, zeta potential, Differential Scanning Calorimetry (DSC), Fourier Transformed Infrared Spectroscopy (FT-IR), and Field Emission Scanning Electron Micrograph (FESEM). Coulter counter analysis serves as a method to choose the best formulation based on the process variables includes different surfactants and its concentration and time of sonication. The formulation A4, B3, C4, D4, and E2 were chosen out of 25 formulations for Malvern zeta sizer based on coulter counter analysis data. Particle size results showed the formulations fall into the category of nanoscales were A4, C4, and E2, while C4 and E2 have excellent stability due to its particle velocity. Based on the particle size and zeta potential resultsE2 has been determined as the optimized formulation. NTG nanocrystals under the optimized conditions gave rise to the mean diameter of 181 nm, zeta potential value of 54 mV and polydispersity index 0.23. DSC results suggest the decreased crystallinity of nanocrystals and its irregular shape of nanoparticles confirmed by the FESEM image. To conclude, nanocrystals are a promising method to improve the solubility of BCS Class II drugs, further in vivo studies are required to claim the therapeutic potentials of Nateglinide nanocrystals.

Key words : Nateglinide;Nanocrystals; Surfactant; Ultrasonication

1. Introduction

Nateglinide is an oral antidiabetic drug that is under the BCS class II which is having low solubility and high permeability. Nateglinide is a non-sulphonyl urea insulinotropic agent, which acts to stimulate insulin secretion when needed( ).Nanocrystal formulation is one of the best among all strategies to improve the solubility of BCS class II drugs. The advantage of this formulation approach is that nanocrystals can help to increase the dissolution velocity and the saturation solubility, which will subsequently increase the bioavailability of Nateglinide(-).For drugs such as Nateglinide, where the oral bioavailability is essential to reduce the glycemic level, it is crucial to obtain a rapid and complete dissolution of the drug.

Drug nanocrystals are the particles consisting only of pure drug with the size range from 200 to 800 nm. The advantage of drug nanocrystals is that it has a high drug loading capacity. Practically the nanocrystals are nanocarriers with 100% drug loading capacity. The drug nanocrystals do not have any matrix material, but they can be stabilized by the addition of the surfactant layer or stabilizing polymer layer. Nanocrystals can be a dispersion in either water or non-aqueous dispersion media, and these dispersions are known as nanosuspension(-).The objective of this study was to formulate the Nateglinide nanocrystals and optimize the process parameters using the ultrasonication method as well as to improve the saturation solubility of Nateglinide adopting the concept of nanonization.

2. Materials and Methods

Materials **:** Nateglinide was purchased from Wuhan Vanz Pharm Inc, China. Poloxamer 188 solutions, Poloxamer 407 and Polyethyleneimine, was obtained from Sigma-Aldrich, Tween 80 was obtained from Chempur, Malaysia and Sodium dodecyl sulphate were obtained from Chemiz, Malaysia.

Selection of surfactant system **:** Atypical strategy includes the addition of hydrophilic polymers and/or surfactants to the nanosuspensions to stabilize nanocrystal formulations. According to previous studies of nanosuspension, hydrophilic non-ionic surfactants such

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as Poloxamer 407 (P407) and Poloxamer 188 (P188), Tween 80 (Tw80), cationic surfactants such as Polyethyleneimine (PEI), and anionic surfactants such as Sodium dodecyl sulfate (SDS) used to prepare nanoparticles and they successfully been incorporated into nanocrystals production to improve the dissolution of those drugs(-).

Drug-excipient compatibility **:** To determine the drug and excipient compatibility, Nateglinide is mixed physically with surfactants, and this physical mixture is then analyzed by using FT-IR to determine if there is any intermolecular interaction between the drug and the surfactants. Fourier transform infraredspectroscopy (ThermoScientific iD5 ATR, Waltham, MA, USA) was used to analyze compatibility between the pure Nateglinide and surfactants( ). A small calibration standard powder was spread on the attenuated total reflection (ATR) crystal and scanned. Between each measurement, the ATR crystal was carefully cleaned with methanol and then air-dried. FT-IR spectra for Nateglinide, surfactants alone and physical mixtures between Nateglinide and the surfactants recorded in the range of 4000-400 cm-1 averaging 20 scans at a resolution of 4 cm-1 and air background using OMNIC software.

Solubility studies **:** The solubility study of the Nateglinide is first determined by adding pure Nateglinide powder in distilled water, methanol, water with 1% SDS. The solution placed in an orbital shaker for 24 hrs. The resulting solution is then subjected to quantification by UV spectrophotometer at 211 nm. The same steps followed for the solubility of Nateglinide in different pH, which is pH 1.2, 6.8, and 7.4( ). The Nateglinide solubility of pH 1.2 assessed in HCl while pH 6.8 and pH 7.4 using phosphate buffer saline (PBS) alone as well as PBS in pH 7.4 with 1% SDS.

Preparation of nateglinide nanocrystals **:** The nanocrystals formulation prepared by adding 100mg of Nateglinide powder dissolved in 5 mL of methanol. The solution then mixed with 0.5%, 1.0% and 5.0% of different surfactants concentration. This mixture placed in the ultrasonicator (Q500 sonicator) for 5 and 15 min at an output power of 500 W ( ). The type of surfactants used, the concentration of surfactants and sonication time was the process variables in this study. The nanosuspension was then lyophilized to get solid nanocrystals for the FT-IR, DSC and FESEM analysis( ).

Saturation solubility **:** The saturation solubility of Nateglinide nanocrystal formulation was determined by adding the nanocrystals into the different solution such as distilled water, distilled water with 1% SDS, phosphate buffer saline, phosphate buffer saline with 1% SDS. The drug content was measured by using UV spectrophotometer (PerkinElmer Lambda 25) (7).

Characterization of Nateglinide nanocrystals

Coulter counter analysis : The particle size of Nateglinide nanocrystals is measured using Coulter counter analysis (Beckman Coulter Z1 Particle Counter, Fullerton, CA) of all prepared formulations. Best formulation from each surfactant was chosen based on the particle size obtained through the coulter counter analysis result.

Zeta sizer and zeta potential : Formulations A4, B3, C4, D4, and E2has been chosen form coulter counter result for the zeta sizer and zeta potential analysis by Malvern zetasizer (Software v 7.03, AA Almelo, Netherlands) at a fixed temperature 25°C.The samples diluted with distilled water before the analysis(8-9). Zeta sizer and zeta potential are measured to find out the average particle size and charge of the Nateglinide nanocrystals. Physically stable Nateglinide nanocrystals should have Zeta potential of 30 mV as a minimum value. Zeta potential of 30 mV is for stabilized nanosuspension in electrostatic form whereas 20 mV is for combined electrostatic and steric stabilization( ).

Fourier transformed infrared (FT-IR) spectroscopy : FT-IR spectroscopy used to investigate the potential intermolecular interaction between Nateglinide and the freeze-dried nanocrystals. The Nateglinide powder and freeze-dried nanocrystals (A4, B3, C4, D4 and E2) were spread on the ATR crystal and scanned. Between each measurement, the ATR crystal was carefully cleaned with methanol and then air-dried( ).

Differential scanning calorimetry (DSC) : Thermal analysis was obtained using a differential scanning calorimeter (Perkin Elmer, USA) for Nateglinide and the optimized freeze-dried nanocrystals E2 formulation. Equivalent to 20mg of sample was crimped in a standard aluminium pan. The thermograms were obtained at a scanning rate of 10°C/min over a temperature range of 30 to 300°C under a constant purging of nitrogen at 40 mL/min (7).

Field emission scanning electron micrograph (FESEM): Scanning electron micrograph of Nateglinide and freeze-dried nanocrystals formulation (E2) was obtained on titanium-coated samples with a Jeol JSM-7600f Schottky Field Emission Scanning Electron Microscope,Tokyo, Japan.

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Statistical analysis:The experimental results are expressed as mean ± SD. Statistical evaluation of the data was done using ANOVA. Statistically, significant differences will be considered at a level of $p < 0.005$.

3. Results and Discussion

Drug-excipient compatibility **:** The FT-IR of the physical mixtures of Nateglinide and all the surfactants are chosen shows that there is no significant interaction between the drug and the surfactants. Therefore, all the surfactant which has been chosen is suitable to be used in this study.FT-IR graph of Nateglinide, PEI and its physical mixture (Fig. 1) shows that the secondary amine functional group of Nateglinide is seen at wavenumber 3248.42 cm-1,Pani N et al.,reported that the (-N-H stretching) is seen at wavenumber 3296 cm-1 according to the characteristic bands of Nateglinide(13-).Other relevant functional groups that were present include (-CH2-cycloalkane) at wavenumber 2925.06 cm-1, the carboxylic acid (-COOH) at wavenumber 1711.85 cm-1 and carbonyl -(C=O) at 1645.33 cm-1 whereas the retaining functional group in polyethyleneimine were the presence of aliphatic methyl (-CH3) at wavenumber 2858.31 cm-1, aliphatic secondary amine (-NH) at wavenumber 1601.67 cm-1 and primary amine carbon (C-N) at wavenumber 1031.45 cm-1which is similar to the study conducted by Hong H et al.,( ).

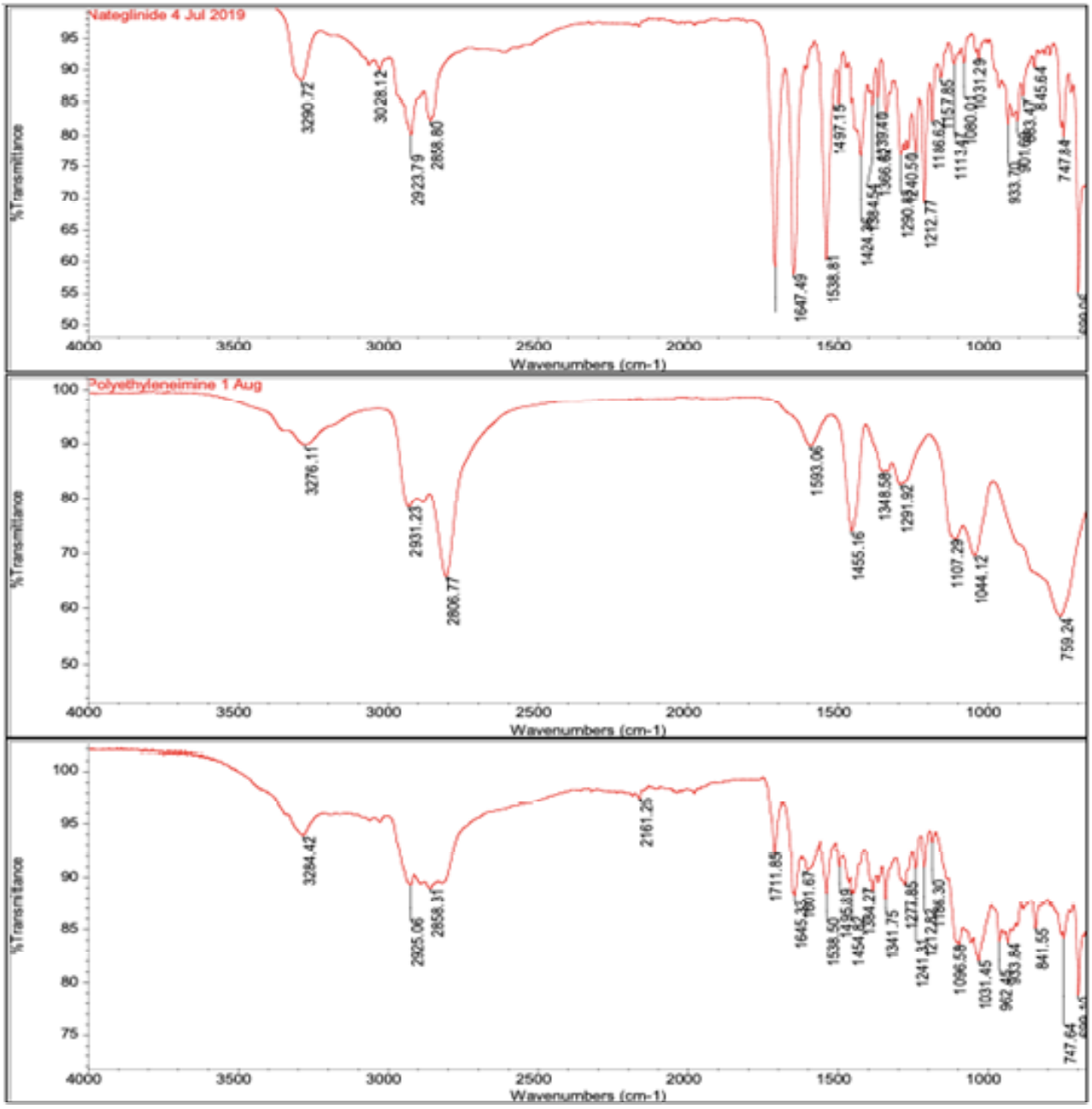

Fig. 1. FT-IR of Nateglinide, PEI and physical mixture of Nateglinide with PEI

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Table 1 : Solubility of Nateglinide in different solve

Solvent	Concentration (mg/mL)
Methanol	95.99 ± 0.7476
Water	0.9492 ± 0.130
Water + 1% SDS	2.205 ± 0.107
PBS	0.8269 ± 0.004
PBS + 1% SDS	0.9868 ± 0.003
pH 1.2	0.09159 ± 0.004
pH 6.8	0.09603 ± 0.001
pH 7.4	0.09982 ± 0.001

***Solubility study* :** Nateglinide is freely soluble in methanol while it is practically insoluble in water( ). FromTable 1, theNateglinide solubility value in water was 0.9492 0.130 mg/mL, which is insoluble. Maggi Let al., revealed this is due to the lipophilic nature of Nateglinide as well as its poor wettability. When Nateglinide powder placed in water, it tends to aggregate, and thereforedifficult to measure its solubility in water( ). Nateglinide has had the highest solubility in methanol (95.99 ± 0.7476 mg/mL),whereas water with the addition of 1% SDS (2.205 ± 0.107 mg/mL) is the second followed by water (0.9492 ± 0.130 mg/mL), PBS with 1% SDS (0.9868 ± 0.003 mg/mL) and phosphate buffer saline (PBS) (0.8269 ± 0.004 mg/mL). Nateglinide has the highest solubility in pH 7.4 that can be seen that (0.09982± 0.001mg/mL) among the different pH ranges.

Preparation of Nateglinide nanocrystals : Nateglinide nanocrystals formulated using various surfactantswith different concentrations at different sonication timesin Table2. The alphabet of each formulation shows the different surfactants, andformulation code A stands for Poloxamer 188; B for Tween 80; C for Sodium Dodecyl Sulphate; D for Poloxamer 407 and E for Polyethyl eneimine. The numbering stands for surfactant concentration and sonication time. The number 1 stands for 0.5% surfactant at 5 min sonication time, number 2 stands for 0.5% surfactant at 15 min sonication time, number 3 stands for 1.0% surfactant at 5 min sonication time, number 4 stands for 1.0% surfactant at 15 min sonication time, and number 5 stands for 5.0% surfactant at 15 min sonication time.

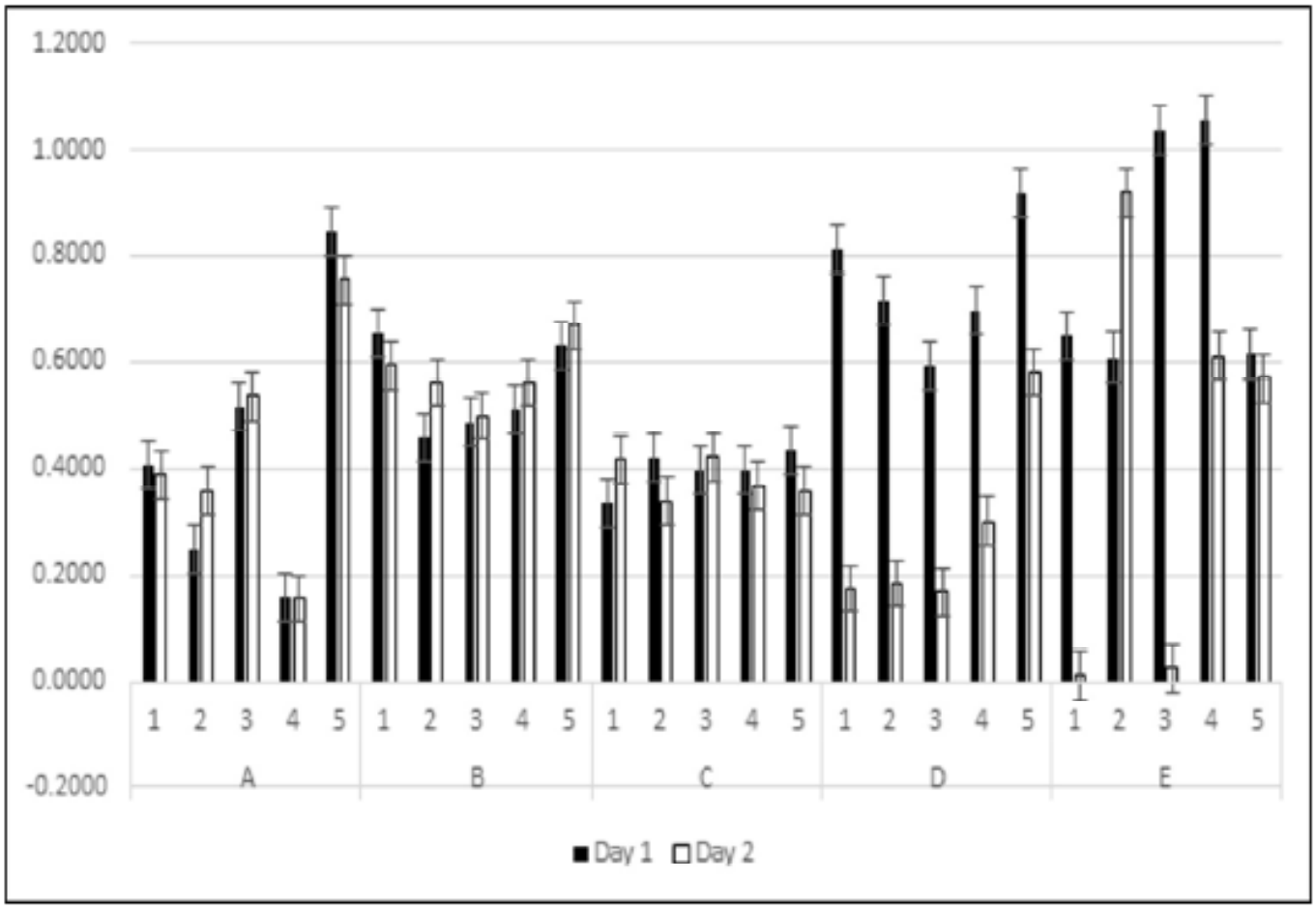

Fig. 2. Saturation solubility of Nateglinide formulation in PBS + 1% SDS

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Table 2 : Formulation code for Nateglinide nanocr

Formulation Code	Surfactant	Concentration of surfactant (%)	Sonication Time (min)
A1	Poloxamer 188	0.5	5
A2	Poloxamer 188	0.5	15
A3	Poloxamer 188	1	5
A4	Poloxamer 188	1	15
A5	Poloxamer 188	5	15
B1	Tween 80	0.5	5
B2	Tween 80	0.5	15
B3	Tween 80	1	5
B4	Tween 80	1	15
B5	Tween 80	5	15
C1	Sodium Dodecyl Sulphate	0.5	5
C2	Sodium Dodecyl Sulphate	0.5	15
C3	Sodium Dodecyl Sulphate	1	5
C4	Sodium Dodecyl Sulphate	1	15
C5	Sodium Dodecyl Sulphate	5	15
D1	Poloxamer 407	0.5	5
D2	Poloxamer 407	0.5	15
D3	Poloxamer 407	1	5
D4	Poloxamer 407	1	15
D5	Poloxamer 407	5	15
E1	Polyethyleneimine	0.5	5
E2	Polyethyleneimine	0.5	15
E3	Polyethyleneimine	1	5
E4	Polyethyleneimine	1	15
E5	Polyethyleneimine	5	15

***Saturation solubility of Nateglinide nanocrystals* :** Nateglinide nanoformulation solubility was done in four different dissolution media such as water, water with 1% SDS, PBS and PBS with 1% SDS.Nateglinide has the highest solubility in buffer saline (PBS) with 1% SDS and pH7.4among different pH ranges. Therefore, PBS pH 7.4 + 1% SDS is chosen as a medium for the dissolution of Nateglinide nanocrystals.Based on Fig. 2, formulation E has the highest solubility overall in PBS pH 7.4 + 1% SDS dissolution media. Remko M,found that if adding 1% concentration of sodium lauryl sulphate of surfactant into water will improve the solubility of Nateglinide. Therefore, it can be seen that formulation E has the highest solubility in day one with dissolution

Table 3 : Coulter counter analysisdata

Sample		Surface mean diameter (μm)	Specific surface area (mm)
A	1	3.661	6.047×10^5
	2	4.236	5.226×10^5
	3	7.422	2.983×10^5
	4	2.716	8.092×10^5
	5	2.735	8.150×10^5
B	1	1.421	1.558×10^6
	2	0.751	2.948×10^6
	3	0.306	7.233×10^5
	4	1.116	1.984×10^6
	5	3.024	7.319×10^5
C	1	3.465	6.389×10^5
	2	4.632	4.779×10^5
	3	5.236	4.228×10^5
	4	2.430	9.109×10^5
	5	4.223	5.241×10^5
D	1	4.312	5.314×10^5
	2	3.283	6.743×10^5
	3	3.319	2.220×10^8
	4	2.651	8.350×10^5
	5	3.401	6.510×10^5
E	1	1.939	1.141×10^6
	2	0.316	6.999×10^6
	3	5.118	4.236×10^5
	4	5.739	3.857×10^5
	5	4.381	5.053×10^5

Table 4 : Results of particle size, zeta potential and polydispersity index

Formulation code	Particle size (nm)	Zeta potential	Polydispersity index
A4	602.56	-9.34	N/A
B3	10.94	-5.5	0.185
C4	272.87	-60.5	0.431
D4	835.31	-4.33	N/A
E2	181.21	55.7	0.230

media PBS pH 7.4 + 1% SDS( ). However, it can be seen that the solubility of formulation E (E1, E3, E4 and E5)has decreased in day 2, except E2. This phenomenon may happen due to the antagonism of surfactants since the surfactant used in formulation E is polyethyl eneimine,whereas the surfactant used in the dissolution is sodium dodecyl sulphate. Antagonism of surfactant will increase surface tension as well as critical micellar concentration leading to a reduction in solubility( ). PEI is a surfactant with a positive charge,whereas SDS is a surfactant with a negative charge.The statistical analysis of one-way ANOVA between 25 formulations showed a

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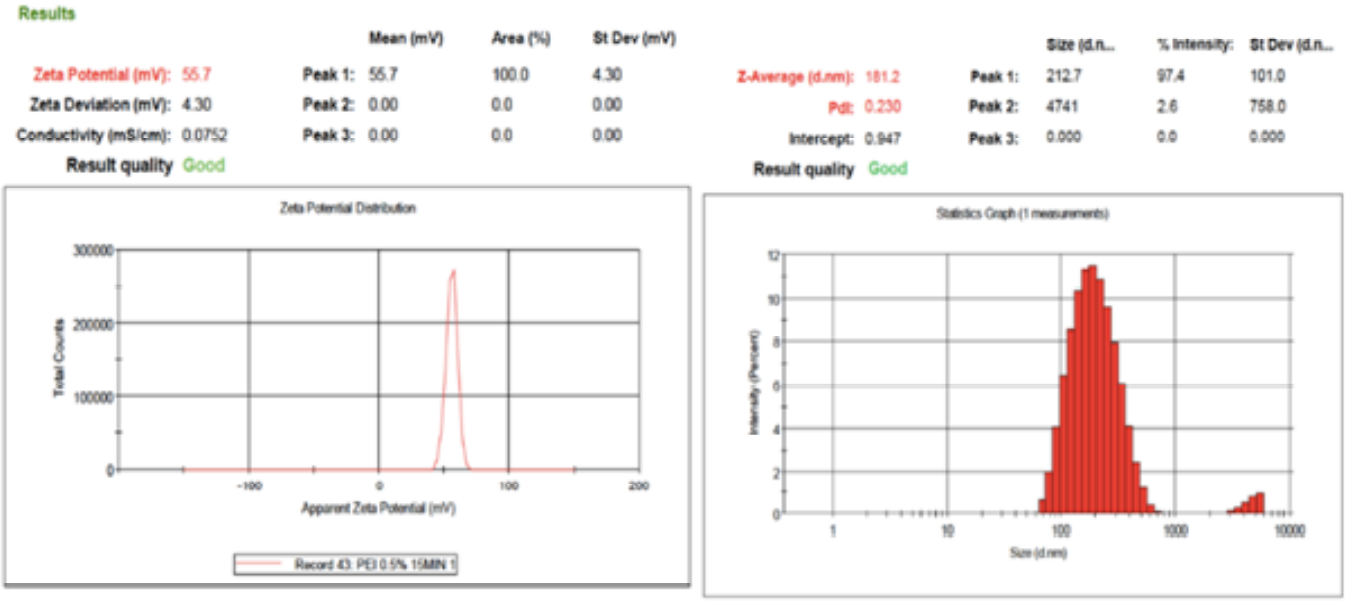

Fig. 3. Particle size, zeta potential and polydispersity index for formulation E2

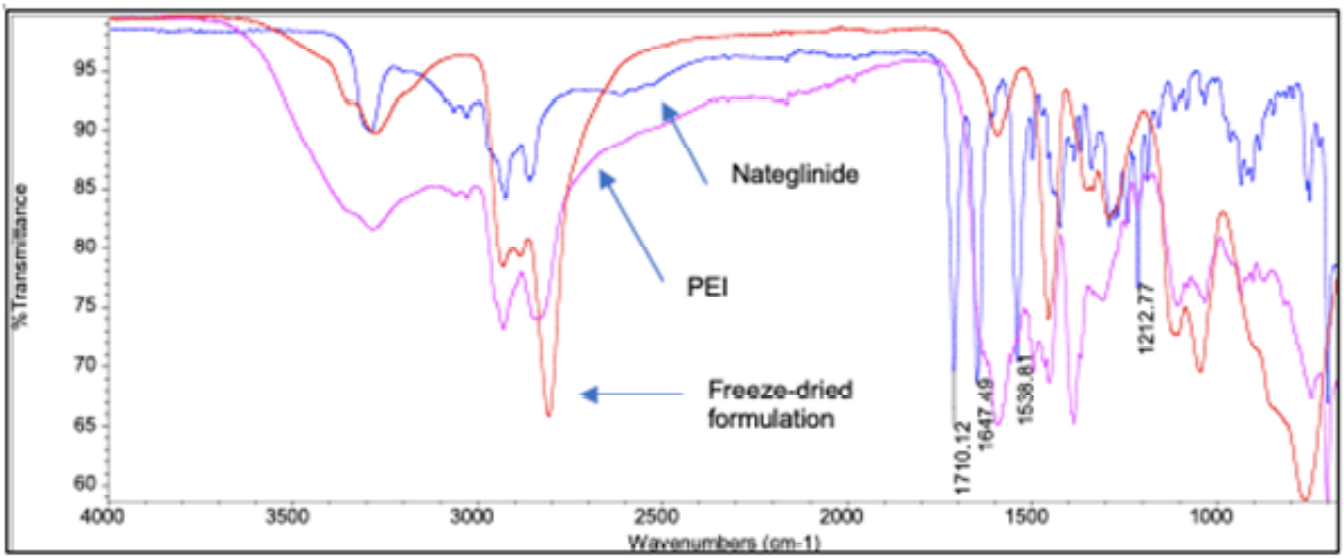

Fig. 4. FT-IR of freeze-dried formulation E2

p-value of <0.005, which indicates that there is a significant difference between the formulations.

Coulter counter analysis **:** The results obtained from coulter counter analysis, formulation A4, B3, C4, D4 and E2 are selected for further analysis. These formulations are chosen based on the smallest particle size among all the formulation from each surfactant concentration. Generally, those formulations are selected as they have the smallest particle size in their surfactant group. From Table3, the selected formulations were highlighted, in whichformulation A4, B3, C4, D4 and E2 had the size of 2.716 m, 0.306 m, 2.430 m, 2.651 m 0.316 m respectively.

Zeta sizer, zeta potential and polydispersity index (PDI) **:** Zeta sizer analysis of the five formulationsare shown in Table 4. Formulation C4 and E2 formulation were fit into the criteria based on their Zeta potential of -60.5 mV and +55.7 mV respectively. The differences between the positive and negative charge may be due to the nature of the surfactant. The results suggest that the addition of the surfactant, which may mask the available charge on the surface of Nateglinide ( ).Formulation C4 consists of sodium dodecyl sulphate, which is an anionic surfactant whereas formulation E2 consists of polyethyleneimine, which is a cationic surfactant.

The last parameter for the selection of the optimized formulation is the polydispersity index (PDI), that would be 0.2 and below required for the formulation of nanocrystals withnarrow size distribution. Low PDI indicates that the nanoparticles exhibita uniform

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distribution of particle size of the nanoparticles and dispersion homogeneity of the nanoparticles ( ).Formulation E2 has a size of 181.21 nm with a zeta potential of +55.7 mV and PDI of 0.230 in Fig. 3, which was prepared polyethyleneimine (PEI) at 0.5% concentration and 15 min sonication time. It has particle size <200 nm with excellent stability to fulfil nanocrystals standards.

FT-IR of freeze-dried formulation **:** FT-IR graph of freeze-dried formulation of Nateglinide with Polyethyleneimine by ultrasonication process does not affect the essential functional groups of Nateglinide and surfactantdepicted inFig. 4. It is evident, with the retention of the functional group at certain characteristic bands ofNateglinide and Polyethyleneimine. The results suggest that the surfactant does not show any significant interaction with Nateglinide even after the ultrasonication as well as the freeze-drying process ( ).

Differential scanning calorimetry (DSC) **:** DSC thermographs of the pure drug is seen in the upper graph in Fig. 5 which exhibited sharp endotherm with a melting point at 140.15°C an associated enthalpy of -94.34 Jg-1. This melting point shows that pure Nateglinide has the polymorph H as the melting point of pure Nateglinide in the H polymorph is around the range of 130 to 140°C. Bruni Get al., discovered that the Nateglinide polymorph H is having a melting point of 137.9 ± 0.5°C (,).The thermal curve of lyophilized Nateglinide nanocrystal showed a broad endothermic effect with 135.52°C and an associated fusion enthalpy of -178.80 Jg-1, indicative of its crystalline state. Endotherm of lyophilized Nateglinidenanocrystal revealed that it had shifted backwards about 5°C with a significant reduction in peak intensity and it may be due to experimental conditions such as ultrasonication speed, moisture content, sample weight and concentration of PEI. It could alternatively indicate that the shift in the melting point shows that there is a physicochemical change to Nateglinide in the Nano-level[18]. A further comparison of X-Ray Diffraction (XRD) of pure drug and nanocrystal formulation of Nateglinide study will be needed to indicate if there are any polymorphic changes in the pure drug after the ultrasonication process( ).

Field emission scanning electron micrograph **:** The scanning electron microscope of pure Nateglinide shows the morphology of Nateglinide as cylindrical sticks in Fig. 6whereas the FESEM of formulation E2 in the lyophilized form showed smaller rod shape that looks

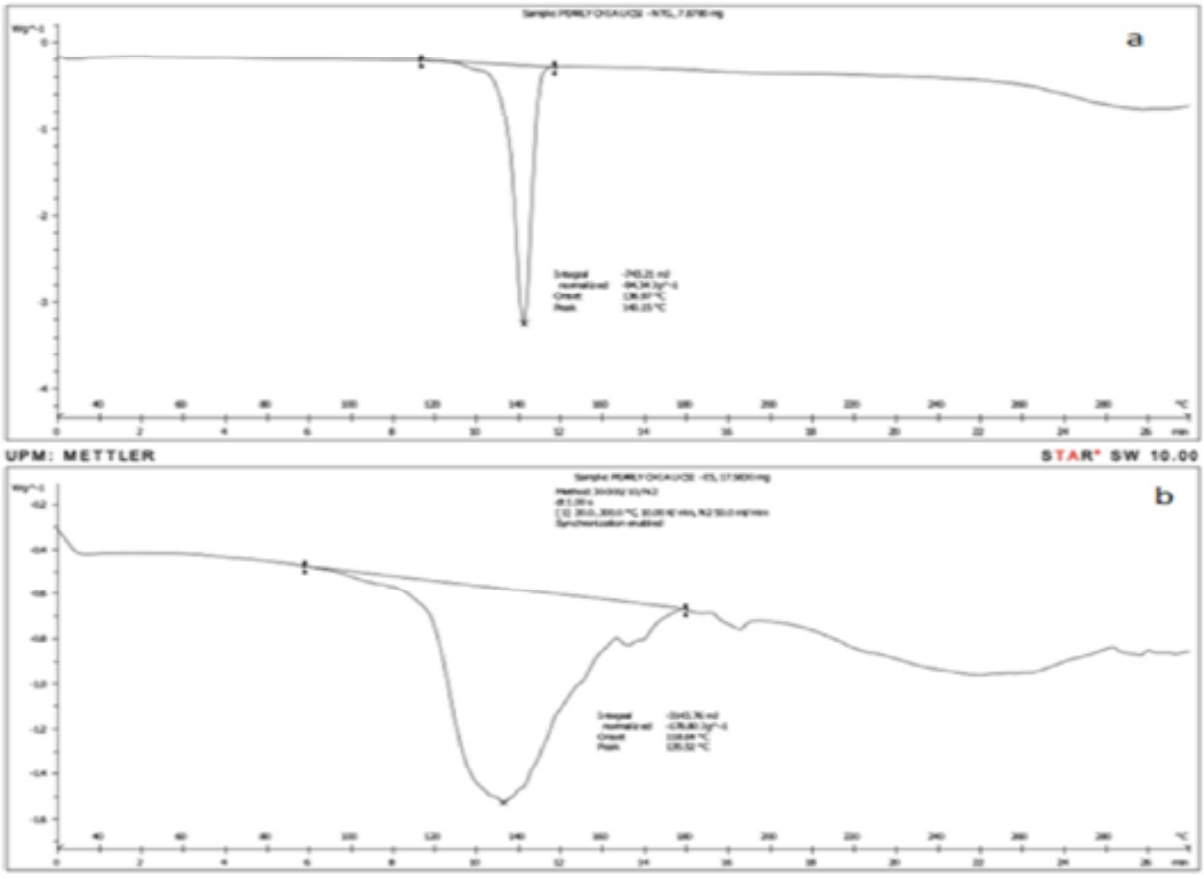

Fig. 5. DSC graph of (a) Nateglinide and (b) Formulation E2

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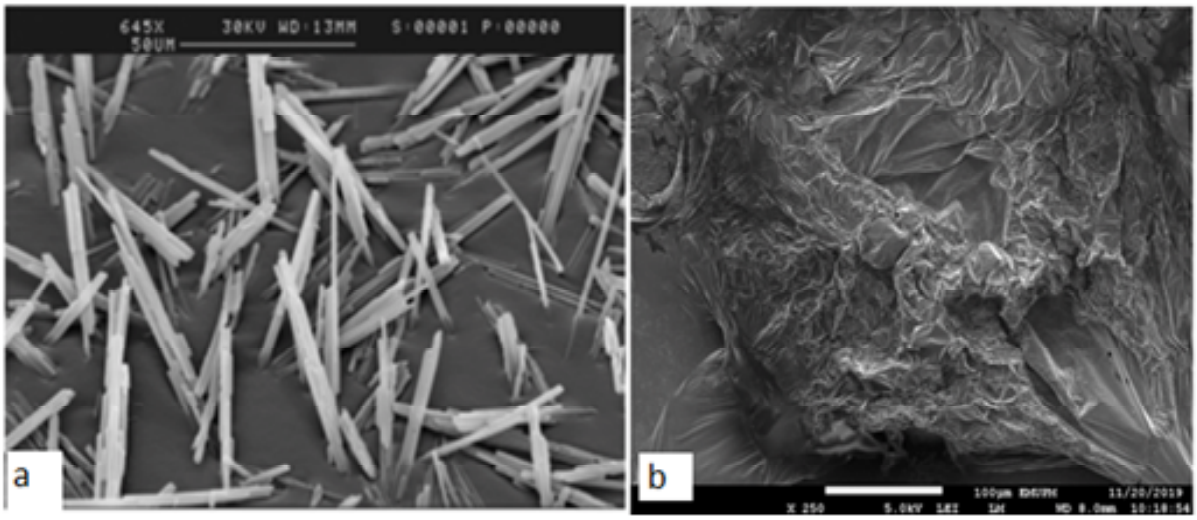

Fig. 6. SEM image (a) Nateglinide and (b) Formulation E2

like aggregate. Kim Het al., reported that the ultrasonication process might cause the nanocrystals to undergo polymorphic changes in their structure[ ].Therefore, the morphology changes of Nateglinide may indicate that there are polymorphic changes in Nateglinide after the ultrasonication process and lyophilization process, but further X-Ray diffraction will be needed to confirm the polymorphism of Nateglinide. XRD can be used to determine if the structure has changed from its crystalline to amorphous form (19).

4. Conclusion

In this study, Nateglinide nanocrystals were prepared by the ultrasonication method with certain process variables. Processvariables manipulated in this studyare the types and the concentration of surfactant used as well as sonication times. Results obtained from coulter counter analysis, five formulations have been selected out of 25 formulations, which were proceeded with particle size measurement using Malvern zeta sizer to analyses the particle size, zeta potential and polydispersity index. Out of five formulations, E2 particle size was 181.21 nm with a zeta potential of +55.7 mV and PDI of 0.230 was optimized for further characterization. The FT-IR of the compounds shows that there is no interaction during the process of ultrasonication as the characteristic bands of each compound are still present. The DSC analysis of the formulation E2 shows that the thermal curve has been shifted 5°C backwards compared to the Nateglinide pure drug. The endothermic peak of 135.52°C indicates that the formulation E2 is in its polymorphic H form.Further study should be conducted with X-Ray diffraction to get a more conclusive understanding of the polymorphic change in the formulations. To conclude, the choice of stabilizers and processing method is vital in achieving the solubility of BCS class II compoundNateglinide. Further, the in vivo studies is required to claim the enhancement in the bioavailability of Nateglinide nanocrystals with optimized process variables.

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The authors have declared no conflict of interest.

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Evaluation of Antibacterial Activity Against Multidrug-Resistance (MDR) Bacteria by the Fractions of *Artabotrys suaveolens* (Blume)

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Abstract

Rising of antibiotic resistance is threatening the global health care system and increasing the worldwide death rate. Therefore, research and discovery of alternative antimicrobial agents from plant sources are encouraged. The *Artabotrys suaveolens* (Blume) belongs to the Annonaceae family and mainly distributed in tropical and subtropical regions of the world. It was indigenously used to treat postnatal weakness and cholera infection.

The study aims to provide evidence of the plant as an alternative source of antibacterial agent based for its folkloric use to treat infection. This study was undertaken to fractionate the chloroform extract of the stem of *Artabotrys suaveolens* (Blume) and to investigate the in vitro antimicrobial activities of different solvent fractions against three ATCC and MDR bacteria.

Liquid-liquid fractionation was performed resulting with petroleum ether, chloroform and water fraction of the stem of *Artabotrys suaveolens* (Blume). Qualitative phytochemical analysis was conducted for alkaloid, cardiac glycoside, flavonoid, saponin, sterol and tannin. Antibacterial activity was ascertained by disc diffusion assay, minimal inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) against three ATCC strains (MSSA ATCC 29213, *K. pneumoniae* ATCC 13883 and *E. coli* ATCC 35218) and three clinical isolated strains (MRSA, *K. pneumoniae, A. baumannii*). GraphPad Prism 8 was used for data analyses. Differences are statistically significant when $p<0.05$.

Qualitative phytochemical analysis revealed that both petroleum ether and chloroform fraction contained alkaloid, sterol and tannin, while water fraction contained cardiac glycoside, saponin, and tannin. Petroleum ether fraction showed notable antibacterial activity against MSSA ATCC 29213 (inhibition zones=10.00±1 mm; MIC=0.5 mg/mL; MBC>2 mg/mL) compared to vancomycin (inhibition zones=10.67±0.58 mm; MIC=0.78 mg/mL; MBC=0.78 mg/mL). It also inhibited MRSA (inhibition zones=11.33±0.58 mm; MIC=0.25 mg/mL; MBC>1 mg/mL) compared to vancomycin (inhibition zones= 11.00±0 mm; MIC=0.78 mg/mL; MBC=0.78 mg/mL), followed by chloroform and water fraction. All three fractions were bacteriostatic against MSSA ATCC 29213 and MRSA based on the results.

The finding in this study has confirmed *Artabotrys suaveolens* (Blume) stem could be an alternative source of antibacterial agent and has provided evidence to the traditional use of *Artabotrys suaveolens* (Blume) in infection. Future studies on the isolation and characterization of bioactive compounds from the fractions are required to confirm their activity.

Key words : Antibiotic resistance, *Artabotrys suaveolens*, bacteriostatic, *Staphylococcus aureus*

1. Introduction

Rising of antimicrobial resistance is threatening the global health care system and increase the worldwide mortality rate, and it is originated from overuse and society abuse of antibiotics (1,2). Anti-microbial resistance would be a global issue in future, that could wipe out 10 million of population by 2050 per year, much more than 8.2 million death from cancer (3). Meanwhile, human has been using the plants for their medicinal purpose for over past decades, due to the precious secondary metabolites which probably possesses many pharmacological effects, inspiring an important element for future drug research and development (4,5). Therefore, research and discovery of alternative and affordable antimicrobial agents from plant sources is encouraged.

The *Artabotrys* genus plants belong to the Annonaceae family, they comprised of more than 100 species worldwide, they are wood-climbing shrubs which distributed mainly in tropical and subtropical regions of

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the world (6), also in tropical region of Africa and Eastern Asia (7). The leaves of *Artabotrys spp* usually arranged as simple, opposite and alternate; textured as coriaceous (leather like), glabrous (smooth surface) and glabrescent (hairless); appeared as glossy (shiny) and the leaves are usually attached to the petiole (stalk). Buds formation at the axils of leaves on the orthotropic branches can grow out vegetative plagiotropic branches, develop into thorns commonly in shady condition or formed sympodial branches with hooks and flowers (8).The flowers are generally white or greenish-yellow colour when ripe, fragrant odour, axillary, solitary, or in clusters of two or three, the peduncles (supporting stalk that bearing flowers) are sharply-hooked shape (9). The 3 sepals (green) and 6 petals (yellow) are usually nearly equal in sizes, free and valvate in aestivation, united and concave at the base. Carpel and stamens are countless and closely arranged, oblong and cuneate (wedge) in shapes. The carpel contains 2 ovules in the ovary. The fruits are cylindrical or ellipsoid and the seeds is oblong shape(8).

The decoction of the roots and barks of *Artabotrys suaveolens* was traditionally used for emmenagogue, and postnatal weakness in Philippines by orally. In Indonesia and India, the infusion and decoction of the leaves of *Artabotrys suaveolens* were orally used to treat cholera (8).

However, the in vitro antibacterial properties of *Artabotrys suaveolens* have not been studied. Therefore, this study is intended to investigate the phytochemical compounds and the antibacterial activity of the water (H_2O), petroleum ether (PE) and chloroform ($CHCl_3$) fractions of the chloroform extracts from the stem of the Artabotrys suaveolens with the aim of establishing an alternative source of antibacterial and the basis for its folkloric use to treat infection (8).

2. Materials and Methods

Plant samples collection

The barks of *Artabotrys suaveolens* were collected from a forest in Perak, Malaysia (4°46'N, 100°56'E). The plant was identified by the FRIM (Forest Research Institute Malaysia). A herbarium sample (PID-251215-10) has been deposited in the FRIM.

Fractionation of plant samples

The chloroform extraction of plant was previously done. 50gm of chloroform extract of *Artabotrys suaveolens* was suspended in 300ml of purified water to make a viscous suspension. Then the suspension was transferred into a separating funnel. 300 ml of petroleum ether was added into the funnel and shaken vigorously with the cap closed to allow partitioning of extract between water and petroleum ether. Two layers of fractions were formed after shaking, petroleum ether fraction layer would be at top and water fraction layer at bottom. Water fraction is collected and transferred into another separatory funnel, while petroleum ether fraction was collected into a round bottom flask (Favorit). To the water fraction, 300ml of chloroform (Merck) was added and shaken vigorously to allow partitioning of extract between water and chloroform. Two layers were formed after shaking, water fraction layer would be at top and chloroform fraction layer at bottom. Chloroform fraction is collected into a round bottom flask (Favorit), followed by water fraction.

The collected petroleum ether and chloroform fractions were concentrated with a rotary evaporator (Buchi, R-200 Switzerland) to remove their respective solvents. Water fraction was freeze dried (Alpha 1-4 LD plus, CHRIST, Germany) to remove water. The concentrated fractions without solvent were carefully recovered and stored in suitable containers, then proceeded to qualitative phytochemical analysis (10,11).

Phytochemical analysis

Qualitative phytochemical analysis of the fractions for alkaloids, cardiac glycosides, flavonoids, saponins, sterols and tannins will be determined as follows: (12-17).

Dragendorff's test for alkaloid **:** 1.7g of Bismuth sub-nitrate, 20ml of Glacial Acetic Acid (GAA), and 5ml of Potassium iodide (KI) solution (50%w/v) are dissolved in water, then make up the volume into 100ml as stock solution. From the stock solution, 10ml is mixed into 20 ml of GAA and make up to 100ml with distilled water as working solution. 2 ml of solution of each fraction was filtered, then the filtrate was mixed with 0.2 ml of GAA in a test tube, followed by 1ml of working solution. Orange-brown precipitate indicates positive of alkaloid.

Keller-Killiani test for cardiac glycosides **:** A mixture of 4.0 ml of GAA and one drop of 2.0% Ferric Chloride ($FeCl_3$) solution were added into 10 ml of plant extract, followed by 1ml of concentrated Sulphuric acid (H_2SO_4). Reddish brown ring appears between the layers confirming the presence of cardiac glycosides.

Shinoda test for flavonoids **:** 4mg of each fraction was dissolved in 2ml of absolute ethanol and filtered. Then the filtrate was treated with 0.5 g of Magnesium turnings followed by a few drops concentrated hydrochloric acid

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(38% HCl). The presence of flavonoids is indicative if pink or magenta-red colour developed after few minutes.

***Froth test for saponins* :** Each fraction was shaken vigorously to froth and was then allowed to stand for 15-20 min and classified for saponin content as follows: (no froth = negative; froth less than 1cm = weakly positive; froth 1.2cm high = positive; and froth greater than 2cm high = strongly positive).

***Salkowski test for sterols* :** 40mg of fraction will be dissolved in 2ml of chloroform and filtered. The filtrate is then added to 1mL of concentrated H_2SO_4. The presence of sterols is indicated by the 2 phases formation with a red colour in the chloroform phase.

***Ferric chloride test for tannins* :** About 1mg of fractions were dissolved in 6ml of hot distilled water and filtered. The solution is divided in two test tubes. To the first test tube, 1ml of 0.9% sodium chloride solution was added. Second test tube was added with 1ml of 0.9% sodium chloride solution and 1ml of 1% (w/v) gelatine solution, and to the third test tube few drops of 2% $FeCl_3$ was added. Formation of a precipitate in the second treatment suggests the presence of tannins, and a positive response after addition of $FeCl_3$ to the third portion which will result in a characteristic blue, blue-black, green, or blue-green colour supports this inference.

Antibacterial susceptibility testing (AST)

The antibacterial activity of plant fraction was evaluated by testing against the clinical isolated bacteria: Methicillin Resistant *Staphylococcus aureus* (MRSA), *Klebsiella pneumoniae* (*K. pneumoniae*) and *Acinetobacter baumannii* (*A. baumannii*) and the American Type Culture Collection strains: Methicillin Sensitive *Staphylococcus aureus* ATCC 29213 (MSSA ATCC 29213), *Klebsiella pneumoniae* ATCC 13883 (*K. pneumoniae* ATCC 13883) and *Escherichia coli* ATCC 35218 (*E. coli* ATCC 35218), with comparison to positive control: vancomycin for Gram-positive bacteria; gentamicin for Gram-negative bacteria, DMSO was used as negative control. All tested bacteria were obtained from local public hospital: University of Malaya Medical Centre (UMMC).

Preparation of bacterial inoculum

The stock culture of each of the six bacterial strains were sub-cultured on Mueller-Hinton agar (MHA) Petri plates at 37°C for 24 hrs prior to inoculation into the Mueller-Hinton broth (MHB) solution.

Few colonies (2 to 3) of similar morphology of the respective bacteria were transferred with a sterile inoculating loop to MHB liquid medium and this liquid culture was incubated at 37°C for 24 hrs. The growth of turbidity of liquid culture was then adjusted equivalent to McFarland 0.5 turbidity standard. The turbidity of the actively growing broth culture was adjusted with sterile 0.9% (w/v) saline solution to obtain turbidity optically comparable to McFarland 0.5 turbidity standard, that indicated the broth culture containing approximately 1×108 CFU/ml for each tested strain(18).

Disc-diffusion test

The tests were performed using Mueller Hilton agar for bacterial strains using disc diffusion method following the Clinical and Laboratory Standards Institute (CLSI) guidelines(19).

100mg/ml of each fraction sample solution were prepared by dissolving 100mg into 1ml of 99.9% DMSO. Then 6mm sterile paper disc was impregnated with 10µL of each sample solution to yield 1mg/disc using micropipette. Noted that all the impregnated paper discs should be completely dried before applying to the agar surface. Each bacterial culture was thoroughly streaked onto the surface of Muller-Hinton agar Petri plates using a sterile swab to ensure complete coverage of the plates and a lawn of growth with uniform thickness. The agar plate was divided into four quarters: three fractions(1mg/disc) and one positive control(1µg/disc). The impregnated 6mm paper disc (1mg/disc) were applied to their respective quarters at approximately equal distance to each other, negative control was placed at the centre of the agar plate. Within 15 min after discs were applied, the agar plates were inverted and incubated with ambient air at 37°C for 24hrs. The antibacterial activity was determined after incubation, by measuring the diameter of zone of inhibition (in mm) include the 6mm disc size. This experiment was repeated as triplicates, and the mean ± standard deviation (SD) of three replicates were presented (18).

Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

The minimum inhibitory concentration (MIC) of the against ATCC reference strains and isolated clinical strains were determined by microdilution dilution, as described by Eloff (20) and using the Clinical and Laboratory Standards Institute (CLSI) as guideline(19).

Stock solution (64mg/ml) of respective plant fraction were prepared by dissolving 64mg into 1ml of 99.9% DMSO. The advantages of using 99.9% DMSO as solvent are elimination of microbial contamination of the plant

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fraction and good solubility during the serial dilution procedures(21). MIC test of the plant fraction against six bacterial strains was aseptically done in 96-well plate as duplicate by two-fold serial dilution method, 100µL of sterile water was prefilled into all the wells of 96-well microtiter plate, followed by 100µL of stock solution (64mg/ml) to yield 50% of the stock solution (32mg/ml) in 200µL after adequate mixing. 100µL from the 50%(32mg/ml) plant fraction solution was transferred to the wells in next row, followed by mixing to yield 25% (16mg/ml) of plant fraction solution. This procedure was repeated for the subsequent 6 rows, resulting in eight concentration ranging from 32mg/ml to 0.25mg/ml in 100 µL solution. Bacterial broth culture with turbidity of 1×108 CFU/ml was adjusted to 100µL of 1×106 CFU/ ml turbidity before adding into wells, finally yielded concentration ranging from 16µg/ml to 0.125mg/ml. The final concentration of positive control is ranging from 25µg/ml to 0.2µg/ml while negative control is ranging from 25% to 0.2%. The microtiter plate was incubated with ambient air at 37°C for 24 hrs. 40 mL of 0.4 mg/ml INT (p-iodonitrotetrazolium, Sigma) INT dye was used to detect bacteria cell viability, pink-purple colour indicates microbial growth. The MIC was visually determined, the lowest concentration that displaying no visible growth (no visible pink-purple colour) was recorded as the MIC (22).

Only fraction that have MIC values of lower or equivalent to 0.5 mg/mL (strong inhibitors) were tested for the MBC values, the content in MIC value and two wells above the MIC value were cultured on MHA. then incubated at 37°C with ambient air for 24 hrs. The MBC was considered as the lowest concentration that produced <10 bacteria colonies after incubation. The plant fraction was considered bactericidal if the ratio MBC/MIC < 4, while considered bacteriostatic if the ratio MBC/MIC > 4(23).

Statistical analysis

The experimental results were expressed as mean ± standard deviation (SD) of three replicates. One-way analysis of variance (ANOVA) and Tukey's testwere employed for the data analysis. P values less than 0.05 were considered statistically significant.

3. Results and Discussion

Fractionation of plant samples

Extraction yield is important to measure the efficiency of solvent to extract desired substances from the raw material (24). Among the three different solvents used for fractionation of chloroform extracts of the Artabotrys suaveolens stem, chloroform provided the highest yield with fractionation yields of 61.92 %. Contrary, petroleum ether provided lowest yield with fractionation yield of 6.73 %. This suggest that the phytochemical components in the stem of *Artabotrys suaveolens* is more favourable to non-polar chloroform (25). Results are showed in Table1.

Table 1 : Percentage yield of fractionation of the chloroform extract of stem of *Artabotrys suaveolens* by various solvents.

Solvents	Yield (%)
Petroleum ether	6.73
Chloroform	61.92
Water	31.35

Qualitative analysis of the P.C. of Artabotrys suaveolens

The results of phytochemical analysis were tabulated in Table 2, water fraction contained cardiac glycoside, saponin, and tannin, while petroleum ether and chloroform fraction contained alkaloid, sterol and tannin.

Table 2 : The results of the phytochemical analysis of the fractionation of *Artabotrys suaveolens.*

Phytochemical constituents	*Artabotrys suaveolens* fractions		
	Petroleum Ether	Chloroform	Water
Alkaloid	++	++	–
Cardiac Glycoside	–	–	++
Flavonoid	+	+	–
Saponin	–	–	+
Sterol	++	+	–
Tannin	–	–	++

The samples were observed for the colours or precipitation produced by the reagent, and the scores would be given accordingly: (-) score: negative, (+) score: weakly positive, (++) score: positive.

Antibacterial Susceptibility Testing (AST)

Disc-diffusion test

First of all, results revealed that all three fractions were showing antibacterial activity against the gram-positive bacteria in this study (MSSA ATCC 29213 and MRSA). Worth to be mentioned that petroleum ether fraction demonstrated best activity against MSSA ATCC 29213 and MRSA. However, no activities were showed against the gram-negative bacteria in this study (*K.*

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Table 3 : Antibacterial activity of the fractions of chloroform extract of *Artabotrys suaveolens* (1 mg/disc) and positive control (1 µg/disc) tested by disc diffusion assay against three ATCC bacteria and MDR bacteria.

Microorganisms	Zone of Inhibition (mm)				
	AS Fractions (1 mg/disc)			Control antibiotics (1 µg/disc)	
	PE	$CHCl_3$	H_2O	Vancomycin	Gentamicin
ATCC strains					
MSSAATCC 29213	10.00±1 [a]	9.67±0.58 [a]	7.83±0.29	10.67±0.58 [a]	NA
***K. pneumoniae* ATCC 13883**	—	—	—	NA	15.00±0
***E. coli* ATCC 35218**	—	—	—	NA	15.00±1
Clinical isolated strains					
MRSA	11.33±0.58 [b]	10.00±0	8.00±0	11.00±0 [b]	NA
K. pneumoniae	—	—	—	NA	16.33±0.58
A. baumannii	—	—	—	NA	—

AS: *Artabotrys suaveolens*, PE: Petroleum ether fraction, $CHCl_3$: Chloroform fraction, H_2O: Water fraction, NA: Not Applicable, and -: no zone of inhibition (0 mm)

Table 4 : Minimum Bactericidal Concentration(MBC), Minimum Inhibitory Concentration(MIC) and MBC/MIC ratio of the fractions of chloroform extract of *Artabotrys suaveolens*(mg/mL) and positive control antibiotics(µg/mL).

Microorganism	AS Fractions MBC/MIC values (mg/mL) MBC/MIC ratio, (+) bactericidal, (–) bacteriostatic			Control antibiotics MBC/MIC values (µg/mL) MBC/MIC ratio, (+) bactericidal, (–) bacteriostatic	
	PE	$CHCl_3$	H_2O	Vancomycin	Gentamicin
ATCC strains					
MSSA ATCC 29213	>2/0.50±00 (–)	>2/0.50±00 (–)	>2/0.50±00 (–)	0.78/0.78±00 1(+)	NA
***K.pneumoniae* ATCC 13883**	NA	NA	NA	NA	0.2/0.2 1(+)
***E. coli* ATCC 35218**	NA	NA	NA	NA	6.25/1.56 4(+)
Clinical isolated strains					
MRSA	>1/0.25±00 (–)	>2/0.50±00 (–)	>2/0.50±00 (–)	0.78/0.78±00 1(+)	NA
K. pneumoniae	NA	NA	NA	NA	0.39/0.20 2(+)
A. baumannii	NA	NA	NA	NA	NA

AS: *Artabotrys suaveolens*, PE: Petroleum ether fraction, $CHCl_3$: Chloroform fraction, H_2O: Water fraction, NA: Not Applicable.

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pneumoniae ATCC 13883, *E. coli* ATCC 35218, *K. pneumoniae* and *A. baumannii*). As expected, no zone of inhibition was produced by the plant fractions against the gram-negative bacteria in this study, because gram-negative bacteria were usually more resistant to plant-derived antibacterial compounds than gram positive bacteria (26, 27). The susceptibility distinction between gram positive and negative bacteria could be explained by their different cell wall structures and composition (28-31).

Minimum Inhibitory Concentration (MIC), Minimum Bactericidal Concentration (MIC) and the MBC/MIC ratio

Since disc diffusion assay has showed only the gram-positive bacteria (MRSA and MSSA ATCC 29213) were susceptible to the plant fraction, and gram-negative bacteria are resistant. Therefore, MIC and MBC test were proceeded for *S. aureus* only. MIC and MBC test were conducted to determine the bacteriostatic or bactericidal activities of plant fractions using 8 mg/mL as the endpoint for MIC, whereby the plant fractions with MIC value at most of 8 mg/mL was showing some significant inhibitory action to the bacteria, any concentrations higher than 8 mg/mL were considered as insignificant or ineffective (32).

Chloroform and water fractions from the chloroform extract of *Artabotrys suaveolens* were showing activity against MRSA and MSSA ATCC 29213 with MIC value of 0.5 mg/mL, except petroleum ether fraction was showing MIC value of 0.25 mg/mL against MRSA. The antibacterial activity of plant fractions was further classified based on the obtained MIC values. MIC values = 0.5 mg/mL and any lower concentrations indicate the plant fractions are strong inhibitors(33). Based on the classification proposed above, the MIC values ranging from 0.25 mg/mL to 0.5 mg/mL by all fractions (petroleum ether, chloroform and water) indicate they were strong inhibitors against MRSA and MSSA ATCC 29213, especially the MIC value of 0.25 mg/mL obtained from petroleum ether fraction against MRSA, suggested its antibacterial activity was double than chloroform and water fractions against MRSA.

Antimicrobial substances were considered bacteriostatic when the MBC/MIC ratio >4, and bactericidal when the MBC/MIC ratio < 4(23). Based on the MBC/MIC ratio, it suggested that all fractions were bacteriostatic against MRSA and MSSA ATCC 29213. Since chloroform and water fractions were inhibiting the growth of MRSA and MSSA ATCC 29213 at 0.5 mg/mL (MIC), but the MBC was probably above 2 mg/mL, thus indicating that MBC/MIC ratio was apparently >4. Worth to be mentioned that petroleum ether fraction alone has inhibited MRSA growth at 0.25 mg/mL (MIC), but the MBC was probably above 1 mg/mL, suggested the MBC/MIC ratio was >4 also.

The zone of inhibition was reported as mean ± SD of three experiments including 6 mm disc (n=3). The values with the same alphabet character indicate there were no significant differences in one-way ANOVA and Tukey multiple comparison test ($p>0.05$)

The relationship between phytochemicals from Artabotrys suaveolens (Blume) and their antibacterial activity

Petroleum ether fraction of *Artabotrys suaveolens* was showing most potent antibacterial activity against MRSA and MSSA ATCC 29213, antibacterial activity was probably due to the presence of alkaloid (34), flavonoids (35) and sterols (36) found in the preliminary phytochemical analysis.

4. Conclusion

Evaluation of the antibacterial activity from the fractions of the chloroform extract of Artabotrys suaveolens (Blume) stem suggested it could be an important bacteriostatic source of alternative antibacterial agent in respect of its selective activity against *S. aureus*. This validates the traditional use of *Artabotrys suaveolens* (Blume) in infection.

Hence, further studies on the isolation and characterization of bioactive compounds from the fractions that responsible for the selective activity against *S. aureus* are required. Further antibacterial studies on other gram-positive bacteria, as well as time-kill assay and synergistic assay are proposed for future studies.

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Conflict of Interest

The authors declare that they have no conflict of interests.

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In-Vitro Cytotoxic Activities of *Brassica oleracea* Var *capitata* by Using Brine Shrimp Lethality Assay

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Abstract

The present study was aimed for phytochemical screening and evaluates cytotoxic activity of ethanol extract of both leaves and seeds of Brassica oleracea var capitata. Both leaves and seed were blended to get the coarse powder and it was subject for extraction by maceration technique. 95% ethanol was used as a solvent for extraction. The cytotoxic activity was determined in terms of its ability to kill the brine shrimps. Both extracts were tested at different concentrations ranging from 10-1000 µg/ml with potassium dichromate served as standard. Cytotoxic assay showing that leaves extract having higher percentage of mortality than seeds extract. Both extracts having moderate cytotoxic activities with LC50 valueof 301.67 µg/ml and 350.76 µg/ml for leaves and seeds, respectively. However, the mortality of brine shrimps caused by both extracts was lesser as compared with standard of potassium dichromate with LC50 value 35.67 µg/ml. The phytochemical screening of both extracts revealed the presence of alkaloids, sterols, terpenoid, carbohydrates, flavonoids, glycosides and saponins. These phytochemicals play a prominent role in cytotoxic activity. Since the plant was showing cytotoxic activity, it can be further subjected for isolation of the therapeutically active compounds with cytotoxic activity and for further pharmacological evaluations.

Key words : Brassica oleracea, phytochemical,cytotoxic.

1. Introduction

Cancer is an abnormal growth of cells which can invade and spread to other part of the body. There are more than 100 types of cancer. Cancer is important leading causes of morbidity and mortality worldwide and poses both economic and psychological challenges. According to World Health Organization (WHO), there are 14 million new cases and 8.2 million of people died each year due to cancer. Cancer contributed to about 13% of all people death worldwide (1). Therefore, cure and prevention of cancer remain a high priority for the scientific community across the world.

Brassica oleracea (B. oleracea) belonging to the member of Brassicaceae family. There are various varieties under Brassica oleracea (B. oleracea) which are B. oleracea var. acephala (kale), var. alboglabra(chinese kale), var. botrytis (cauliflower), var.capitata (cabbage), var. gemmifera (Brussels sprouts), var. gongylodes (kohlrabi), var. italic (broccoli) and var. sabellica (Curly and Portuguese kale). Cabbage is native to Coastal Southern and Western Europe (2). It is the common vegetable we consumed everyday which are inexpensive and rich in nutrients.

Cabbage is use as medicine since ancient time. In folk medicine cabbage was used to cure hangover, sore throat, abscess, and dysentery. Cabbages also used to cure simple and complicated injuries, rheumatic pains, facial neuralgia, headaches, leg ulcer, anthrax, and many others. During World War I, they were used to treat trench foot of soldiers. Moreover, the water in which cabbage leaves have been cooked has been used to treat rheumatism (3).

During recent decades, there has been an increasing demand for finding newer and safer chemotherapeutic agents. Besides, the current diseases are increasingly become resistant to the drugs. Numerous studies have shown thatvarious natural products can interfere with different stages of cancer which are cancer induction, growth and progression therefore may effectively block malignancy (4). Thus, it is important to discover new drugs to combat with drug resistant and adverse side effects ofsynthetic drugs.

The present study was undertaken to investigate cytotoxic activity of leaves and seeds extract of this plant.

2. Materials and Methods

Materials

95% ethanol, sodium chloride, sodium bicarbonate,

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potassium dichromate, Fehling A reagent, Fehling B reagent, chloroform, concentrated sulphuric acid, concentrated hydrochloric acid, iron (III) chloride, Wagner's reagent, and sodium hydroxide.

Preparation of plant materials

Fresh cabbage and dried seeds were purchased from local market in Cheras. After through washing, the leaves were cut into small pieces and dried completely under shade at room temperature. The dried leaves were ground to coarse powder by using blender.

Extraction of plant material

The extracts were obtained by using cold maceration method. The powder was soaked in 95% ethanol. Periodical stirring of the mixture was performed for the next 48 hours. The extracts were filtered, and the extraction was repeated twice (5). Then, the filtrate obtained was evaporated at 60°C by using water bath.The extract was then dried and stored in desiccators. A semi-solid extract was formed.The resultant yield of extract obtained was 14.78% and 10.67% of dry weight for leaves and seeds, respectively.

Preliminary phytochemical screening

Phytochemical screening were performed using standard procedures (6, 7, 8).

Test for alkaloids

Wagner's test **:** small quantity of extract was dissolved in 8ml of 1% HCl and warm for two minutes. It was filtered and few drops of Wagner's reagent was added. Formation of reddish-brown precipitate indicates the presence of alkaloids.

Test for steroids, terpenoids and cardiac glycosides:

Salkowski test **:** Small quantity of extract was dissolved in 5ml of chloroform. It was filtered and 2 ml of concentrated H_2SO_4 was added to form a layer. The formation of brown ring indicates presence of sterols.

Test for carbohydrates

Fehling's test **:** Small quantity of extract was dissolved in 5ml of water and filtered. Equal volume of Fehling A and Fehling B reagents were mixed. The aqueous extract was added to boiling Fehling's solution in a test tube. A brick red coloured precipitate of cuprous oxide forms indicates the presence of carbohydrates.

Test for flavonoids :

Alkaline reagent test **:** A Small quantity of extract was dissolved in 5ml of water and filtered. Extracts were treated with few drops of diluted sodium hydroxide solution. Formation of intense yellow colour, which becomes colourless on addition of dilute acid, indicates the presence of flavonoids.

Test for phenols :

Ferric chloride test **:** A Small quantity of extract was dissolved in 5ml of water and filtered/ Extracts were treated with 3-4 drops of 10% ferric chloride solution. Formation of bluish black colour indicates the presence of phenols.

Test for saponin :

Foam test **:** A Small quantity of extract was dissolved in 10ml of distilled water. The solution was shaken vigorously and observed for a stable persistent froth for 20 min.

Test for tannins :

A Small quantity of extract was boiled in 10 ml of water in a test tube and then filtered. A few drops of 0.1% ferric chloride was added and observed for brownish green or a blue-black colouration.

In-vitro cytotoxic activity

Brine shrimp lethality bioassay was carried out to investigate the cytotoxicity of extracts of B. oleracea var capitata. Artificial sea water was prepared by dissolving 38gm of NaCl in 1 liter of distilled water for hatching the shrimp eggs. Small quantity of sodium bicarbonate was added to obtain the pH 8.4 as sea water. The seawater was put in a small plastic container (hatching chamber) with a partition for dark (covered) and light areas. Shrimp eggs were added into the dark side of the chamber while the lamp above the other side (light) was used to attract the hatched shrimps. Oxygen was supplied through an air pump. Two days were allowed for the shrimp to hatch and mature as nauplii (larva). The crude extract was dissolved in artificial sea water to obtain 10mg/ml as stock solutions. Then, a series of solution of varying concentrations (10µg/ml, 50µg/ml, 100µg/ml, 500µg/ml and 1000 µg/ml) were prepared from the stock solution by serial dilution method. Ten nauplii were drawn through a capillary tube and place in each well containing 4.5ml artificial sea water and added various concentrations of crude extract. The final volume was made up to 5 ml using artificial sea water. A parallel series of tests with the standard potassium dichromate solution (10-100 µg/ml) were tested and the blank control was always included. After 24 hours, surviving shrimps in each vial were viewed with a magnifying glass, counted and the

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survival data recorded (9). Six replicates were used for each treatment and control. The percentage of mortality (% M) is calculated as (10).

%M=number of dead nauplii/total number of nauplii×100

In case where negative control deaths occur, the data were corrected using Abbott's formula (11):

% M= [(test - control)/control] x 100

3. Results and Discussion

Preliminary phytochemical screening

Table 1 showed the result of phytochemical screening of leaves and seeds extracts of Brassica oleracea var capitata. Both extracts indicated the presence of the following secondary metabolites; alkaloids, sterols, terpenoid, carbohydrates, flavonoids, glycosides and saponins. The phenols and tannins were found to be absent in both extracts. According to previous research, the principle active compounds detected here which include alkaloids, sterols, terpenoid, flavonoids and saponins are known to poses cytotoxic activity of the brine shrimps (12,13).

Table 1 : Qualitative phytochemical analysis of ethanol extracts from leaves and seeds of Brassica oleracea var capitata.

Phytochemical components	Ethanol Extract	
	Leaves	Seeds
Alkaloids	+	+
Sterols	+	+
Terpenoid	+	+
Carbohydrates	+	+
Flavanoids	+	+
Phenols	-	-
Glycosides	+	+
Saponins	+	+
Tannins	-	-

(+) Present, (-) Absent

Tab 2 : In-vitro cytotoxic activity of potassium dichromate and Brassica Oleracea var capitata extracts.

Serial No.	Groups	Concentration (µg/ml)	% of mortality Mean ± S.D. (n=6)	LC_{50} (µg/ml)
1.	Potassium dichromate	10	21.67±7.53	35.67
		20	61.67±4.08	
		40	71.67±7.53	
		80	80.00±8.94	
		100	93.33±5.16	
2.	Leaves extract	10	25.00±5.48	301.67
		50	33.33±8.17	
		100	58.33±7.53	
		500	75.00±8.37	
		1000	96.67±5.16	
3.	Seeds extract	10	20.00±8.94	350.76
		50	40.00±6.32	
		100	51.67±7.53	
		500	65.00±8.37	
		1000	91.67±7.53	

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In-vitro cytotoxic activity

In the present study, BSLA was used to evaluate cytotoxic activities of B. oleracea leaves and seeds extract. BSLA is an efficient, rapid and inexpensive assay for testing biochemical activity of plant extract (14). It was useful for preliminary screening the plant extract's toxicity(15). LC50 value lower than 1000 µg/ml is considered bioactive in toxicity evaluation of plant extracts by BSLA11. Values of LC50 between 500 µg/ml to 1000 µg/ml are categorized as weakly cytotoxic, those between 100 and 500 µg/ml as moderately cytotoxic, and those less than 100 µg/ml was considered to have strong cytotoxic activity (16).

The result of cytotoxic activity of ethanol extract of B. Oleracea and positive control potassium dichromate in terms of mortality of brine shrimps were presented in Table 2 and graphically present in figure 1, 2a and 2b.There are varying degrees of lethality observed with exposure to different concentrations of the test samples in brine shrimp lethality assay (BSLA). The degree of lethality was found to be directly proportional to the concentration of the extract. Increasing in concentration of test samples increased mortality gradually. The maximum mortalities took place at the highest concentration of 1000µg/ml whereas the least mortality took place at 10µg/ml in the evaluation for cytotoxicity using BSLA. In this investigation, both leaves extract and seeds extract exhibited cytotoxic activities with the LC50 values 301.67µg/ml and 350.76 µg/ml respectively. These showed that both seed and leaves extract having moderate cytotoxic activities. Besides, the leaves extract having the highest percentage of mortality when compared with seeds extract. However, the mortality of brine shrimps caused by both extracts was lesser as compared with potassium dichromate which serves as a standard. When compared LC50 of leaves and seeds extracts, leaves extract is more potent than seeds extract.

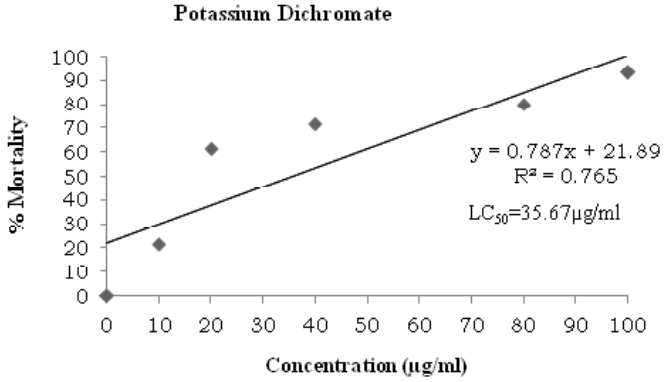

Fig 1 : The toxicity effects of the potassium dichromate using brine shrimp lethality assay after 24 hours.

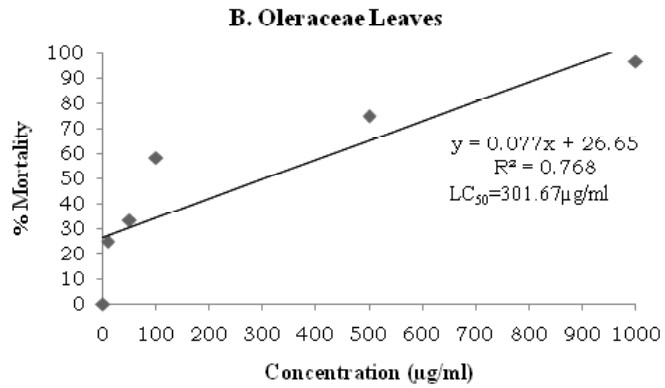

Fig 2a : The toxicity effects of the Brassica Oleracea var. capitata (leaves) using brine shrimp lethality assay after 24 hours.

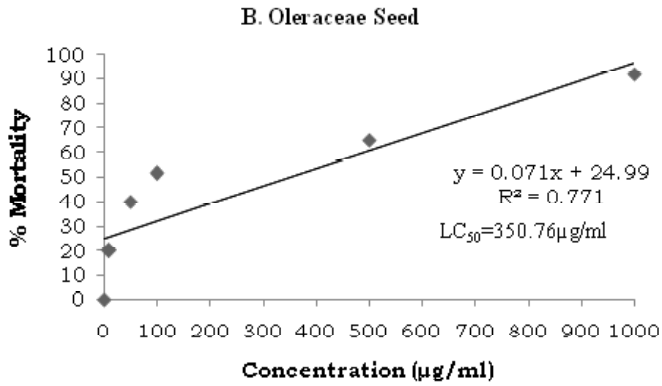

Fig 2b : The toxicity effects of the Brassica Oleracea var. capitata (seeds) using brine shrimp lethality assay after 24 hours.

The cytotoxicity of the extract is contributed by presence of alkaloids, flavonoids and terpenoids(12). Moreover, the presence of saponins in extract also justified the potency as antitumor and anticancer agent(13).

Alkaloid has been shown to exhibit antiproliferation and antimetastasis activities on various types of cancers both in vitro and in vivo (17) . Flavanoid has been shown to exhibit antimutagenic and antimalignant effects (18). This was associated with flavonoid can interfere with the initiation, development, and progression of cancer by the modulation of cellular proliferation, differentiation, apoptosis, angiogenesis and metastasis (19). Terpenoids have been shown to suppress the growth of a variety of cancer cells without exerting any toxicity in normal cells. Terpenoids act at various stages of tumor development, inhibit initiation and promotion of carcinogenesis, induce tumor cell differentiation and apoptosis, and suppress tumor angiogenesis, invasion, and metastasis through

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regulation of various transcription and growth factors as well as intracellular signaling mechanisms (20). Saponins are natural glycosides characterized by their strong foam-forming properties in aqueous solution (21). A study reported that saponin derived from plant revealed anticancer effects against both human lung cancer cell NCI-H460 and human breast cancer cell BT474 (22) and cytostatic activity towards Hep G2 (human hepatocellular carcinoma), HEK293 (human embryonic kidney epithelial cell line) and MCF7 (human breast carcinoma cell line) (23).

4. Conclusion

This study concluded that both B.oleracesleaves and seeds extract revealed qualitative presence of alkaloids, sterols, terpenoid, carbohydrates, flavonoids, glycosides and saponins. On the other hand, both 95% ethanol extract of B. oleracea leaves and seeds having moderate cytotoxic activity with LC50 of 301.67µg/ml and 350.76µg/ml respectively but the cytotoxic activity was lesser as compared with potassium dichromate. In conclusion, the plant might have potential to develop as a useful and safe cytotoxic alternative and further study on different pharmacological activities and isolation of active ingredients could provide leads to interesting pharmaceuticals of plant origin.

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