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Scoping Review on Health Literacy and Its Effect on Medication Adherence in Chronic Disease Patients

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Abstract

Health literacy (HL) is an important aspect of various literacies in the field of health and education. Medication adherence is important for effective treatment regimens and managing several health conditions.Scoping review was conducted to define health literacy and its effects on medication adherence among chronic disease.Articles from PubMed and Google Scholar with selected keywords were selected for review.Inclusion criteria were adults aged 18 and above with chronic diseases in the community.Data was collected and reviewed by two researchers. All included articles were summarized to understand the relationship between health literacy and medication adherence among patients with chronic diseases. A total of 13 out of 18153 articles were found and included in this review. Three articles defined health literacy, and ten articles were reviewed for the correlation between health literacy and medication adherence in patients with chronic diseases. Out of the ten articles, four focused on non-specific chronic diseases, three on type 2 diabetes mellitus, and three on hypertension. Understanding health literacy's definition can affect medication adherence. Health literacy together with medication adherence can be affected naturally, and interventions are necessary to improve them. Individual differences also play a role in health literacy and medication adherence.The relationship between health literacy and also medication adherence heavily affects individuals with chronic diseases.

Keywords: health literacy; medication adherence; chronic diseases; non-communicable diseases.

Introduction

Patients today have to manage complex self-care routines and take different medications, but medications taken inappropriately can lead to serious problems. This is especially true if patients do not understand their health information well. Research shows that over 300 million people struggle to understand health-related information. Misunderstandings can lead to lower awareness of illness, worse health outcomes, and more hospital visits(1). In 2016, the National Survey on the Use of Medicines (NSUM) in Malaysia surveyed consumers. The results showed that different factors influenced people's choice to buy medications from clinics. These factors include age, ethnicity, job, education level, and monthly income(2).

Non-adherence to medical and drug recommendations is a significant public health issue as postulated by Ngoh. The phrases *adherence* and *compliance* are interchangeable, although their implications differ. *Adherence* suggests the patient agrees with the instructions, whereas *compliance* implies passiveness(3). Medication compliance, often referred to as medication adherence, is the extent to which a patient follows their doctor's instructions and suggestions when taking medication. For treatment regimens to be effective and for a variety of health issues to be managed, medication adherence is necessary. Non-adherence can result in worsening symptoms, inefficient therapy, higher medical expenses, and a decline in general well-being.

Malaysia's National Health Morbidity Survey of 2015 revealed that the

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country's incidence of chronic diseases is rising. The largest incidence of non-communicable diseases nationwide was determined to be hypercholesterolemia, which was followed by hypertension and diabetes mellitus (4). According to the National Survey on the Use of Medicines (NSUM) by Malaysian Consumers in 2016, of the respondents, about 30.3% said they were taking chronic medications. There emerges a significant and well-researched correlation between medication adherence and also health literacy in the context of chronic diseases. An important consideration in the treatment of chronic diseases mostly in the elderly is medication adherence. Age-related changes in medication adherence as well as behaviours towards medication (5). Interventions are needed to improve health literacy in chronic disease settings. Studies have identified factors contributing to poor medication adherence, but more research is required to focus on health literacy as the primary factor. Effective therapies to enhance adherence are in demand with the increase in self-administered medicines. Recent studies aim to understand the complex obstacles that influence patient adherence(6).This scoping review study investigated health literacy and its effects on medication adherence in chronic disease patients to understand medication adherence in people who self-administer medication.

Methods & Materials

The Preferred Reporting Items for Systematic Reviews and Meta-Analyses Extension for Scoping Reviews (PRISMA-ScR) Checklist criteria are used in reporting this scoping review (7).

During November and December of 2023, a thorough search of the literature was conducted on Google Scholar and PubMed. The focus of the search was to gather relevant articles, surveys, and journals related to medication adherence, health literacy, chronic diseases, and patient hypertension, diabetes mellitus, and cardiovascular disease. The search only included peer-reviewed journals published in English. The search terms utilized were carefully selected to match the present study design and included qualitative, interview, survey, questionnaire, systematic review, and meta-analysis.

An example of search strategy in PubMed as follows: ("health literacy"[MeSH Terms] OR ("health"[All Fields] AND "literacy"[All Fields]) OR "health literacy"[All Fields]) AND ("effect"[All Fields] OR "effects"[All Fields]) AND ("medication adherence"[MeSH Terms] OR ("medication"[All Fields] AND "adherence"[All Fields]) OR "medication adherence"[All Fields]) AND ("chronic disease"[MeSH Terms] OR ("chronic"[All Fields] AND "disease"[All Fields]) OR "chronic disease"[All Fields])) AND ((ffrft[Filter]) AND (2010:2024[pdat]).

Inclusion and exclusion criteria

This study reviewed published research on health literacy together with medication adherence in the context of chronic diseases from a community perspective. The inclusion criteria were limited to articles, surveys, and journals related to common chronic diseases in Malaysia, published in English within the last 20 years. The study only focused on adults aged 18 years or older with chronic diseases and excluded articles on mental health conditions, children and adolescents under 18 years, and publications unrelated to research objectives or common chronic diseases in Malaysia.

Study selection

We conducted a thorough search across 13 publications to identify pertinent articles, surveys, journals, or systematic reviews. We then removed any duplicates and screened titles and abstracts to determine eligibility. Relevant articles were subject to a full-text review, and reviewers regularly discussed eligibility criteria. Our focus was on describing the effect of health

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literacy on medication adherence among chronic disease patients, and thus, no risk of bias was assessed.

Data extraction and synthesis

Firstly, we defined health literacy by identifying crucial details, such as the author, publication year, and definition used in various articles. Data was collected by two researchers using a Microsoft Excel template. We reviewed and assessed the data for quality, and then synthesized the findings both qualitatively and narratively. Finally, we summarized articles, surveys, and systematic reviews from the ten included articles, focusing on the relationship between health literacy and medication adherence in patients with chronic diseases.

Results and Discussion

Figure 1 shows the process of selecting articles. A total of 18153 articles were found from PubMed and Google Scholar, out of which 13 were included in the review. Three duplicates were removed before the screening process. Articles that were excluded in this stage were 64, with various reasons as follows: irrelevant to studies, wrong population, wrong diseases, wrong outcomes, wrong language, unrelated title, wrong medications, and study protocol. The articles included varied study designs and analyzed both quantitative and qualitative data. The review covered three articles on health literacy's definition and ten articles on the connection with medication adherence in patients with chronic diseases, with four articles on nonspecific chronic diseases, three on type 2 diabetes mellitus, and three on hypertension.

Definitions of health literacy

Understanding the concept of health literacy is vital in effectively communicating messages, promoting health equity and patient empowerment, conducting research, developing policies, clinical practice, and public health programs. In a 2020 study on the Influence of Health Literacy on Medication Adherence Among Elderly Females with Type 2 Diabetes in Pakistan, health literacy

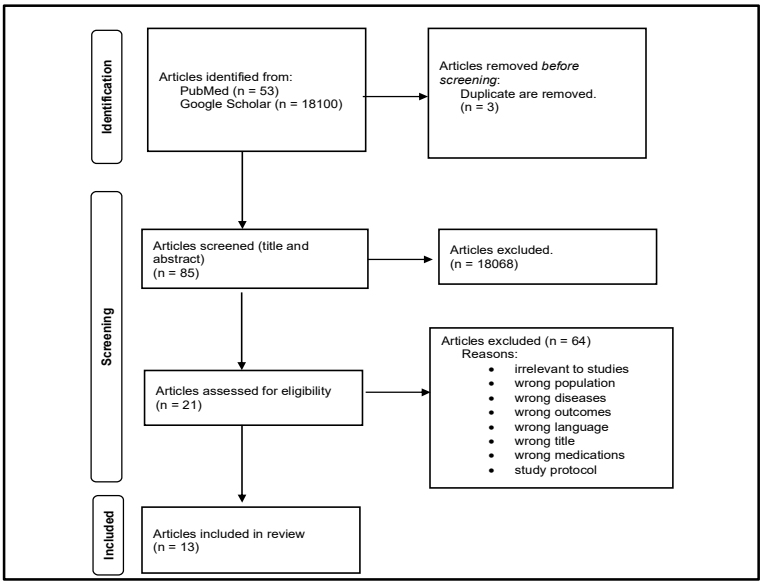

Figure 1: Flow diagram of the article selection process

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was defined as "the skills of reading and writing (also known as print literacy). It also includes speaking or listening (oral literacy), conceptual knowledge, and the ability to apply numbers as needed to manage health (quantitative literacy or numeracy)." Hussain et. al study revealed important differences in medication adherence between those who met the criteria for adequate health literacy and those who did not, indicating the impact of health literacy on medication adherence

Another 2020 study on Health Literacy and Health Beliefs with Adherence to Antihypertensive Medications in an Urban African American Cohort defined health literacy as "the ability to understand and act on a physician's instructions, is related to morbidity and mortality from a variety of conditions" which the opinions of participants on the importance of exercising, getting prescriptions filled for antihypertensive medications, and maintaining a healthy diet were indicative of their health literacy. In line with previous studies, the study discovered that health literacy was an important indicator for medication adherence (8).

To address the lack of a widely agreed-upon definition for the term "health literacy," a systematic review was conducted in 2012 titled "Health Literacy and Public Health: A Systematic Review and Integration of Definitions and Models" where 17 different definitions focus on a person's capacity to access, process, and understand health-related information and services to make well-informed decisions about their health. The authors defined health literacy as "people's knowledge, motivation, and competencies to access, understand, appraise, and apply health information to make judgments and take decisions in everyday life concerning healthcare, disease prevention, and health promotion to maintain or improve quality of life during the life course" (9).

The effects or relationship of health literacy on medication adherence in chronic disease patients

In this review, we examined 10 studies that investigated the connection between health literacy with medication adherence among individuals with chronic illnesses across 6 different countries. From the 10 studies, 4 were conducted in the United States, 2 in China, and 1 each in Switzerland, Japan, Taiwan, and Malaysia. The studies primarily centered on prevalent chronic conditions of type 2 diabetes and hypertension. Out of the 10 studies, 6 revealed a strong interrelation between health literacy and medication adherence. Conversely, 3 studies did not demonstrate a clear connection, while 1 showed no effect.

Many research studies the connection between chronic disease patients' medication adherence and health literacy. Younger individuals who experience side effects are more likely to be unintentionally non-adherent(10). Medication adherence can be enhanced by health literacy and social support (11). There exists a positive relationship between health literacy with medication adherence(12,13). In addition, a studyrevealed a positive relationship of health literacy and medication adherence and a negative relationship between treatment burden and medication adherence(14). Text messages improve patients' health literacy levels and medication adherence(15).

Health literacy indirectly affects medication adherence through factors such as medication self-efficacy and health status(16). Someimpacts of health literacy are on refill adherence(17). Medication adherence was inversely associated with health literacy, but only after controlling for cognitive ability(18). There was improved compliance to antihypertensive medication associated with higher health literacy, but self-reported levels of adherence to antihypertensive medications and health literacy were both low(19) (Table 1).

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Table 1: Summary of Scoping Review for Health Literacy and Medication Adherence in Chronic Disease Patients

Study	Study Design	Concept	Results	Illness	Country
(Guo et al., 2023)	Community-based cross-sectional study	Structural equation modelling (SEM) is used to look into the interrelationship between latent variables such as health literacy and medication adherence.	Health literacy not only directly affects the level of medication adherence among hypertension patients, but also indirectly mediates the relationship of medication adherence with age, education level and marital status.	Hypertension	China
(Náfrádi et al., 2016)	Cross-sectional survey	Assess the socio-demographic, clinical and psychological determinants of intentional and unintentional non-adherence	Health literacy is shown to be revealed as a positive determinant of intentional medication adherence	Hypertension	Switzerland
(Yeh et al., 2018)	Cross-sectional survey study	Evaluate the relation between disease-specific health literacy, disease knowledge and adherence behaviour of patients with type 2 diabetes.	Critical health literacy includes numeracy skills and using this information to make further decisions. It resulted in the respondents having poor numeracy skills which included medication adherence as a numeracy item. Low health literacy worsens diabetes as it is associated with adherence behaviour	Type 2 Diabetes Mellitus	Taiwan

(*Contd.*)

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Table 1: Summary of Scoping Review for Health Literacy and Medication Adherence in Chronic Disease Patients (*Contd.*)

Study	Study Design	Concept	Results	Illness	Country
(Miller, 2016)	Quantitative meta-analysis	Study of the relation between patient health literacy and both medication adherence and non-adherence with health literacy interventions effects.	The average relationship between health literacy and patient adherence suggests positive and highly significant, thus increasing both health literacy and treatment adherence	Chronic diseases	United States
(Selvakumar et al., 2023)	Cross-sectional study	Assess effects of treatment burden with health literacy towards medication adherence among older adults with multiple chronic conditions (MCC)	This resulted in a significantly negative correlation between treatment burden and medication adherence, but a significantly positive correlation between health literacy with medication adherence.	Chronic diseases	Malaysia
(Sugita et al., 2017)	Single-centre, open-label, randomized controlled study	Evaluating text message-based health literacy intervention promoting medication adherence compared to text message reminders only	Text messages affect medication adherence which leads to improvement of the patient's health literacy levels	Type 2 Diabetes Mellitus	Japan

(*Contd.*)

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Table 1: Summary of Scoping Review for Health Literacy and Medication Adherence in Chronic Disease Patients (*Contd.*)

Study	Study Design	Concept	Results	Illness	Country
(Huang et al., 2018)	Cross-sectional study utilized a face-to-face survey	Associating health literacy and medication self-efficacy with self-reported diabetes medication adherence	After adjusting other variables, initial data revealed health status and medication self-efficacy related to diabetic medication adherence, although health literacy was not. Health literacy may not be directly related but it influences drug adherence.	Type 2 Diabetes Mellitus	United States
(Gazmararian et al., 2006)	Prospective cohort study	Evaluating the relation between health literacy and medication refill adherence with medications used for cardiovascular disease.	Health literacy resulted in a moderate effect on refill adherence but overlapped with the null hypothesis when controlling other factors. No association between health literacy and refill adherence but not rule out the relation to medication adherence.	Chronic diseases	United States

(*Contd.*)

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Table 1: Summary of Scoping Review for Health Literacy and Medication Adherence in Chronic Disease Patients (*Contd.*)

Study	Study Design	Concept	Results	Illness	Country
(Jia et al., 2022)	Cross-sectional survey	Looking into the relationship between health literacy and medication adherence and intervention of cognitive ability among older adults with chronic disease.	Health literacy was negatively associated with medication adherence but when the cognitive ability factor was controlled, the association was still found between health literacy and medication adherence.	Chronic diseases	China
(Lor et al., 2019)	Cross-sectional survey	Evaluate the correlation of health literacy level and antihypertensive medications adherence	Higher health literacy associated with better adherence, but both health literacy and self-reported antihypertension medication adherence levels are low among study participants.	Hypertension	United States

Health literacy definition affects medication adherence

Effective health management involves two key components: medication adherence and health literacy. Health literacy encompasses the capability to access, comprehend, and apply fundamental health information and services. This includes reading and comprehending medication labels, interpreting dosage instructions, and navigating the healthcare system. As patient involvement in decision-making becomes more valued, health literacy plays an increasingly important role. Although health literacy has been defined in a variety of ways, it typically refers to a person's proficiency in locating, comprehending, and utilizing health information in a streamlined manner.

Nature effects on health literacy and medication adherence

Older adults with low health literacy face higher risk of hospital admissions, worse self-management abilities, and

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problems with medication adherence. Health literacy can affect medication adherence based on sociodemographic factors, and age is a prevalent factor. Older patients are more likely to use adherence aids, while younger participants report higher levels of unintentional non-adherence due to lifestyle factors.

Approaches in enhancing health literacy and medication adherence

Medication adherence efforts are useful for enhancing health literacy. Text messages encouraging medication adherence are particularly useful. By involving patients in clear communication techniques, we can enhance their understanding of treatment regimens, prescription guidelines, and potential side effects, leading to improved health literacy. Comprehensive health literacy interventions can address a wide range of health-related knowledge and skills, while also considering individual needs and varying levels of health literacy.

Individual differences affect health literacy and medication adherence

The consequence of health literacy on medication adherence in patients with chronic diseases may be influenced by several factors, including the intricate nature of individual health behaviors and variances. Past research indicates that individuals with low level of health literacy encounter difficulties with medication adherence and comprehending their treatment plans.Additional studies are necessary to discern the full extent of this issue. This scoping review offers valuable perspectives on health literacy definitions, factors that impact medication adherence, and can aid in developing effective treatment approaches.

Conclusion

The relationship between health literacy and medication adherence heavily affects individuals with chronic diseases. Those who possess a high level of health literacy are better equipped to comprehend prescription instructions, which ultimately reduces the occurrence of medication errors. Understanding the importance of health literacy is crucial for various sectors such as research, policy creation, clinical practice, public health initiatives, and patient empowerment. Furthermore, patients who have a heightened level of health literacy adhere more to their prescribed medication regimen. As such, health literacy has a significant role in medication adherence for chronic disease patients and can be improved through various factors. Researching medication adherence interventions can prove to be highly beneficial for patients dealing with chronic diseases, particularly the elderly. With the elderly being commonly affected by chronic conditions and being prescribed multiple long-term medications, this research can shed light on the various factors that impact medication adherence and aid in addressing the challenges faced by this demographic in following their recommended prescriptions.

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Systematic Literature Review on Alternative Options For Halal Critical Ingredients In Halal Pharmaceutical and Cosmetics

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Abstract

The global halal market is expanding fast as halal products establish themselves as a new standard for safety and quality assurance. The constituent of a product, whether pharmaceutical or cosmetic, determines its halal classification. Ingredients that do not correspond to the halal standard are commonly known as critical ingredients. As a result, various substitutes for critical ingredients should be developed to raise global demand for the halal market. This study aims to review the current research development on the alternatives for halal critical ingredients in halal pharmaceuticals and cosmetics and to explore the testing methods used to test the alternative option for halal critical ingredients. This systematic study followed the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) standards and all the publications in this review meet the research eligibility requirements, which were searched and selected using electronic databases such as PubMed, Scopus, and MyCite. This study examined approximately 21 publications that proposed various substances derived from sources such as plants, animals, marines, and microbes. Three publications illustrated the possible use of the choice in insulin-resistant patients, whereas seven articles discussed on the potential replacement for gelatine. Insulin and gelatine are the most frequently explored topics among the publications included in this review. Their alternative possibilities, whether derived from plants, marine sources, or microbe-based substances, are thoroughly evaluated to determine their desired effect or activity. The testing methodologies demonstrated that the alternative possibilities are far superior to the critical ingredients in terms of texture, morphology, activity, composition, and even the cost of synthesis.

Keywords: halal; critical ingredients; systematic literature review; insulin; gelatine

Introduction

The Arabic word "halal" implies "permissible" or "lawful". According to the law of Islam, halal refers to any act, way of life, or even object that is regarded as permissible or authorized, whereas "haram" refers to any action or way of life that is deemed prohibited or interdicted. Halal and haram refer to any product used by consumers, such as food, cosmetics, and medicinal products.

In an article written by Mustafa[1], the halal worldwide industry is fast expanding as halal-certified products receive widespread recognition as they set a new standard for safety and quality. Goods that conform to halal norms and rules, regardless of type, can simply receive a halal certificate from the authorities. As a result, when a halal-certified product is presented to the market, consumers accept it with confidence, particularly Muslim consumers, who have religious considerations. Because Muslims

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make up the vast majority of Malaysia's population, the halal market has surely grown into a global industry. The halal industry is a new concept in the twenty-first century; therefore, to adapt to the changes, producers take advantage of chances to make a product with improved strategies and innovations to certify it as halal.

Because of technical advancements, the halal concept now includes pharmaceutical and cosmetic products in addition to food products. In a publication by Siddiqui[2], the concept of Halal in pharmaceuticals especially in the global market is something new which is not surprising given the embryonic state of Halal supply chain and logistics management in general. Global Islamic Economy Summit (2013) states that halal has evolved largely into three categories: Food, Cosmetics, and Pharmaceuticals [2]. Even though halal pharmaceuticals are quite new Nain [3]elaborates that halal pharmaceuticals are gaining popularity, with the global Halal pharmaceutical business valued at approximately US$800 billion per year.

In Malaysia, the authorities, Jabatan Kemajuan Islam Malaysia (JAKIM), established a standard known as the Malaysian Standard – Halal Pharmaceuticals and Halal Cosmetics, which serve as a guide for manufacturers to design a halal product. They must also comply with the requirements to facilitate the halal certification procedure of cosmetics and medicinal items. The halal status of a product, whether pharmaceutical or cosmetic, is determined by the ingredient used because it determines the efficacy and safety of the product. As a result, the ingredients must be certified halal before being used in a product composition. According to Sugibayashi [4], substances that do not correspond to the halal system are frequently referred to as critical ingredients. Thus, to determine their halal certification, their properties must be investigated as well as their efficacy if employed in a product. If the critical ingredient is halal, it can be incorporated into the formulation to facilitate the product's halal certification. Furthermore, halal items may also see an increase in demand among non-Muslims if the halal critical ingredients are more effective and safer than non-halal critical ingredients.

However, there are several obstacles in determining the halal status of the critical ingredient. This is because most producers were unwilling to provide extensive information about their products, and the halal status of the critical ingredient has yet to be determined. To overcome these challenges, authorities in charge of Halal products must address them, explain the importance of Halal, and enforce the law governing product formulation to ensure that manufacturers use the appropriate ingredients according to the Halal Standards and Guidelines. Therefore, this systematic literature review provides more insights into the alternatives for halal critical ingredients in halal pharmaceuticals and cosmetics so that the halal market not just broadens in food but also in cosmetics and pharmaceuticals.

Methodologies

Research design

This study's design is a Systematic Literature Review (SLR). SLR is a type of literature review that uses a systematic and rigorous process to discover, analyze, and synthesize all available research related to a certain research issue or topic. In SLR, research questions are created and then conducted a systematic search for studies or previously published papers that were appropriate for our study. The publications will be locatedusing specificsearch phrases and parameters in predetermined databases. To avoid outliers in data collecting, inclusion and exclusion criteria are used while searching for relevant papers. The data-gathering procedures are pre-defined and stored in an Excel sheet, which can then be utilized to support our

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findings and discussions. After collecting relevant and suitable papers, it will beanalyzed and summarized to reach a conclusion based on our research questions and objectives. The major concern of this systematic literature review is to provide a high-quality and comprehensive study that includes all the relevant previously published papers used in this investigation. In this systematic literature review, keywords relating to systematic literature reviews will be entered into each database's search column. Boolean operators "AND" will be used to combine keywords to narrow down certain search titles. Meanwhile, "OR" will be utilized to combine the terms to broaden the search titles.

Preferred reporting items for systematic reviews and meta-analyses (prisma)

PRISMA refers to Preferred Reporting Items for Systematic Reviews and Meta-Analyses. It is an evidence-based minimal set of things to report in systematic literature reviews and meta-analyses. PRISMA was selected as one of the data-collecting and analysis methods because it establishes a widely accepted standard for reporting evidence in systematic literature reviews and meta-analyses.

Inclusion and exclusion criteria

Inclusion and exclusion criteria were developed before proceeding with the systematic literature review study as they are essential to make sure all applicable data are presented properly. The inclusion criteria that are included in this study are the publication of the article should be in either Malay or English language and the publications should be ranged from the year 2013 until 2023. In addition to that, original research studies only were selected to maintain the quality of the study. On the other hand, the exclusion criteria are the publication's language other than English and Malay language and the publications that are not related to the research questions and objectives. Publications that are related to review articles were also excluded from this study.

Study selection

Articles that showed in the database results after conducting keyword searches were carefully checked and eliminated if they were duplicated. The recovered articles were then carefully examined so that they met the inclusion and exclusion criteria that were predetermined and specified at the start of the investigation. Following an assessment of each publication, the articles that met the eligibility standards were incorporated into this research. The reasons for the excluded articles were briefly stated in the PRISMA flow chart (Figure 1).

Data extraction

The key information from the included articles was extracted and tabulated, as shown in the results and appendices. Examples of information extracted include the author, year of publication, database source, country, research methodology used in the study, summary of the article, types of journals, critical ingredients in pharmaceuticals and cosmetics, alternatives for the critical ingredients, testing methods for halal authentication, and study conclusions.

Critical appraisal

Quality assessment is a critical stage in completing a systematic literature review. This is because it will aid in determining the risk

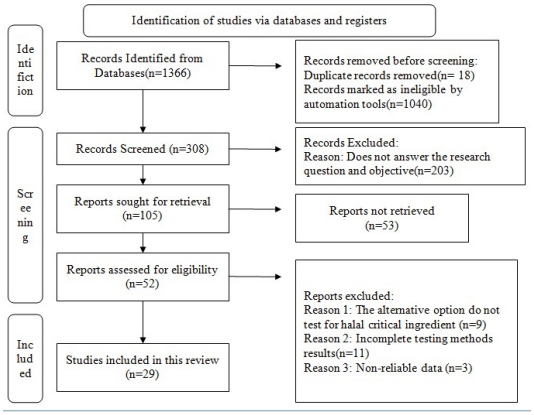

Figure 1: PRISMA Flow Diagram

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of bias in particular studies and overall trust in the review findings. To conduct a quality assessment, a standard form should be produced and given to reviewers for use in evaluating each publication[5]. The standard form is commonly referred to as an assessment tool for reviewing research papers and determining the validity of the study mentioned in the article. There are various types of tools based on the study design. In this review study, JBI Checklist appraisal instrument was selected for the quality assessment of the papers, which are based on the research methodology of the study.

Results and Discussion

Article selection and screening process

The article searches yielded 1366 articles from four databases. Table 1 presents an overview of the search results. A total of 308 papers were screened for prospective inclusion in this study. In comparison, the remaining 1058 publications were removed from this study since they did not meet the inclusion criteria and the records were duplicated. Approximately 207 articles were excluded because they were not retrievable from the database. To finish the search results, 29 publications were selected and included in this review study during the last stage of the data selection procedure. Among the 29 articles, there are 8 articles achieved through PubMed, another 9 collected through MyCite, and 12 from Scopus. Figure 1 demonstrates the selection of the articles through the PRISMA flow diagram.

Descriptive statistics

A descriptive analysis was conducted on the retrieved papers used in this study. Analysis, such as the number of publications by year, the number of papers published by publisher nation, the research methodology employed in the study, and the number of publications by journal type, were conducted and sorted accordingly. According to the number of publications by year, 2018 had many papers published as shown in Figure 2A. This could be attributable to an increase in global demand for halal products. As a result, researchers and producers work together to make halal products that are more dependable and of higher quality. Before the year 2018, there were not many papers written since the halal concept was a new idea in the cosmetic and pharmaceutical sector therefore there was not much demand for halal cosmetic and pharmaceutical products. In terms of the number of articles published based on the country of publisher, Malaysia had the most based on Figure 2B. This is because Malaysia has a larger Muslim population than other countries, therefore halal has become a primary issue in their daily lives, resulting in a rise in halal-related research. Based on Figure 3A experimental type research was discovered to be the most common in the research methodology employed by the gathered publications because the study focuses on critical substances, hence testing for an ingredient must be done experimentally. Finally, the number of publications by journal type revealed that journals classified as others published many papers linked to the study. The other type of publication has a large number of conference papers from many mediums; thus it cannot be divided into the types of journals as shown in Figure 3B. Five of the included publications indicated important chemicals that are commonly used in the beauty and pharmaceutical industries. This research employed a variety of approaches to determine the halal status of the key ingredient. Magnesium stearate's halal certification was verified using FTIR and chemometric analysis as shown in Table 2. However, the halal status remains dubious because the acquired FTIR data show no substantial difference between the various magnesium stearate sources[7]. On the other hand, three articles have shown that collagen and allantoin are important elements. Even though allantoin was synthesized, the process of istihalah was unable to occur due to many factors, making allantoin's halal

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Table 1: Critical ingredients commonly used in the pharmaceutical and cosmetic industry (*Contd.*)

No	Themes	Sub-themes/ Supporting Evidence
1	Pharmaceutical	Magnesium Stearate - The halal status of magnesium stearate was conducted by chemometric investigation. The results showed that magnesium stearate from animal sources such as bovine is not entirely halal whereas plant and kosher sources are halal based on the peaks of chemometric [7]. - The halal status of magnesium stearate was conducted by FTIR study. In this study we couldn't make a conclusion on the halal status of magnesium stearate as there were no prominent peaks of the FTIR raw spectra, hence their halal standards remain unclear[7].
		Enzyme (Trypsin in Insulin) - A study showed that insulins from animals that have been slaughtered according to Islamic law are considered halal however if it's a non-halal animal source then the insulin is not permissible[8].
2	Cosmetic	Keratin - Keratin is a type of protein that can be found in human hair and soybeans. Hence, the protein from the human hair or non-permissible animal is considered non-halal [9].
		Hyaluronic Acid - Hyaluronic acid is considered forbidden according to the Syariah law if the sources are from animals and humans because it is commonly present in the ocular fluid and the fetus [9].
		Allantoin and its derivatives -Found in various biological materials so allantoin is found to be haram if they derived from humans or animals [9]. - Typically, the extraction of allantoin from plant sources requires alcohol. Even though, the source of the substance is halal the extraction process makes the halal status of allantoin doubtful [10]. - According to the Islamic perspective, synthetic allantoin is halal, as is allantoin obtained from animal urine through several processes such as oxidation and hydrolysis, which do not change the chemical properties of the ingredients allantoin even though their physical properties change. This prevents the process of istihalah from occurring. Because of this, its halal status remains unclear [10].

(*Contd.*)

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Table 1: Critical ingredients commonly used in the pharmaceutical and cosmetic industry (*Contd.*)

No	Themes	Sub-themes/ Supporting Evidence
		Collagen - Animal-derived collagen does not conform to the halal system, whereas plant-based collagen is made from hydrolyzed wheat proteins and contains an extension composed of hydroxyproline, a similar amino acid residue to that found in mammals that contributes to collagen properties. As a result, it is halal but not as effective as animal-derived collagen [11]. - Is a connective tissue present in various biological materials. Haram whether it comes from people or animals [9].
		Protease Enzyme - This can be acquired from either animals or plants. Halal if derived from plants [11].
		Riboflavin - Colour; Halal if derived from synthetic sources. Otherwise, it requires further inquiry to determine its source [11].
		Fast Yellow AB - It is a chemical colour that is Halal when used as a dry powder; however, its liquid form is only Halal when Halal solvents are utilized [11].
		Mono Starch Phosphate - Phosphate from animal bones is not halal; however, phosphate from minerals is halal[11].
		Quinoline Yellow - Colour. It is a chemical colour that is halal when used as a dry powder. Its liquid form is only Halal when Halal solvents are used [11].
		Patent Blue V - Colour. It is a dry petroleum basis. It is halal when used as a powder colour. When Halal solvents are employed, the liquid dye is Halal [11].
		Carmine - Colour. It was once harvested from plants, but it is now synthesized synthetically. It is Halal if it is made synthetically from Halal ingredients. The liquid form is Halal if the solvents used are Halal [11].
		Chlorophyll -Colour. It is a plant pigment that is Halal if the extraction solvents employed are Halal rather than alcohol [11].
		Copper Complex of Chlorophyll - Colour. It is a plant pigment that is Halal if the extraction solvents employed are Halal rather than alcohol [11].

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Table 1: Critical ingredients commonly used in the pharmaceutical and cosmetic industry (*Contd.*)		
No	Themes	Sub-themes/ Supporting Evidence
3	Cosmetic and Pharmaceutical	Gelatin - There are two varieties of gelatin. Type A is derived from non-halal animals, usually pigs. This form of gelatin is acid-processed and can create large quantities of high-quality gelatin, whereas form B gelatin is derived from the bones or skin of cows and buffaloes and is alkaline-treated. Because the halal status for both types does not conform to the halal system, it requires full research from the Islamic perspective [8].
		Glycerin and its derivatives - It can be obtained naturally or synthetically. If the source of the substance from haram animal fat then it is considered not permissible [9]. - If it is from an animal source (bone), it is classified as mushbooh according to the E code, indicating that further clarification and study are required [11].

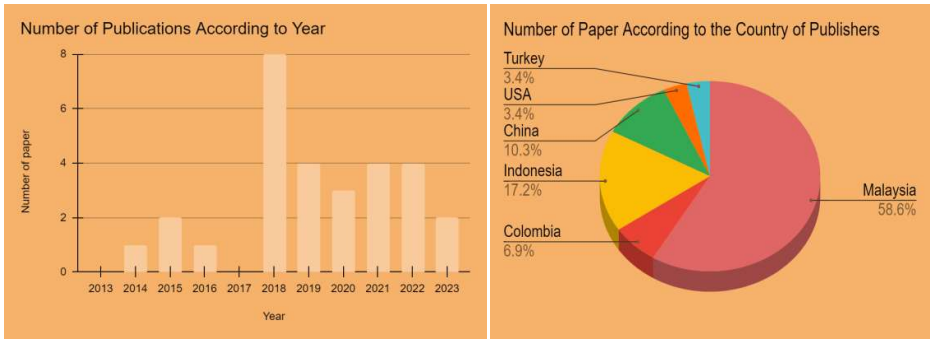

Figure 2: Number of published articles based on (A) year throughout 2013–2023 and (B) publication distribution according to the country of origin

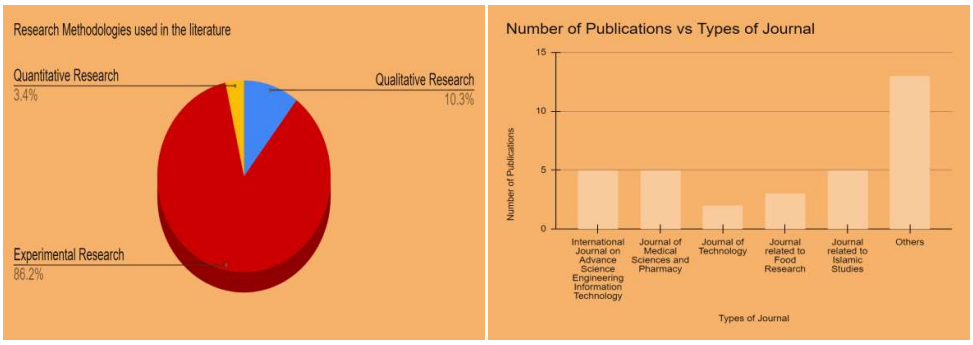

Figure 3: Number of published articles based on (A) research methodologies and (B) Different types of journals used

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Table 2: Alternative options for halal critical ingredients used in pharmaceuticals and cosmetics

No	Themes	Sub-themes/ Supporting Evidence
1	Plant-based Ingredient	Okara - Acts as a substitute for important ingredients in cosmetics. Okara oil contains a high concentration of functional lipids, making it a viable alternative source of essential oil for cosmetic purposes. Okara showed excellent potency as a functional cosmetic ingredient, primarily for improving skin conditions, acting as a skin whitening agent, and providing UV ray protection [12].
		Gum Arabic - Alternative to gelatine, a versatile hydrocolloid. The results revealed that the optimal formulation in terms of physicochemical properties, antioxidant activity, and sensory acceptability was a sample containing 12% gum Arabic and 4% gelatin. Thus, gum Arabic is an appropriate alternative to gelatine [13]
		Aquilaria malaccensis Leaf - An alternative to nonsteroidal anti-inflammatory drugs (NSAIDs) and steroids. The study found that (Gas chromatography-mass spectrometry) GCMS of leaf (supercritical fluid extract) SFEX revealed a peak of a tricyclic sesquiterpene that was tested for possible analgesic and anti-inflammatory effects [14].
		Pea Protein Isolate -An alternative to gelatin. The results showed that at a protein concentration of 9-16%, thermo-reversible gels were generated, with several of them having strong gelling properties and increasing the pH up to 4.2. The pea protein gel is translucent, thermo-reversible, and has strong mechanical properties, making it suitable for substituting gelatin [15].
		Plant-based Cellulose - An alternative to commercial cellulose. The Fourier Transform-Infrared (FTIR) demonstrated the usual peaks of conventional cellulose. The results revealed that cellulose produced from Borassus flabellifer fruit peel fibres had remarkable similarities with the conventional cellulose employed and a 12.4% of large cellulose yield was produced [16].

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Table 2: Alternative options for halal critical ingredients used in pharmaceuticals and cosmetics (*Contd.)*

No	Themes	Sub-themes/ Supporting Evidence
		Flower Extracts - An alternative to commercial sunscreens with titanium dioxide or zinc oxide as active components. The UV filtering effectiveness of flower extracts from Rosa centifolia L., Posoqueria latifolia (Rudge) Schult, and Ipomoea horsfalliae was evaluated. The P. latifolia extract was shown to be the most promising for use as a sunscreen due to its significant photoprotective efficacy, antigenotoxicity, photostability, and comparatively low cytotoxicity and genotoxicity in human fibroblasts [17].
		Bolanthus spergulifolius (Caryophyllaceae) - An alternative to synthetic insulin. The data imply that extracts of B. spergulifolius promote programmed cell death, enhance lipid excretion, and, up-regulate glucose transporter levels, and may leads to increasing insulin sensitivity in DM [18].
		Acer truncatum leaves -An alternative to synthetic insulin. Myricitrin which is the active component can repair aberrant mRNA sequence of pro-inflammatory cytokine in diabetic conditions, mostly via interfering with toll-like receptor pathways so that they can improve glucose absorption and reduce blood glucose level in the body. [19].
		Curcumin - An alternative to synthetic steroids. Curcumin consumption was found to suppress the development of hypersensitivity and anaphylaxis reactions in susceptible mice.Th2 responses were similarly decreased, and curcumin inhibited mast cell activation. The study found that curcumin decreased the activation of the pro-inflammatory gene induction in susceptible mice [20].
		Moringa Oleifera Leaves -Alternative to collagen, which is frequently utilized as an active ingredient in facial mask formulations. The results revealed the percentage of protein content in conventional facial mask samples is lower compared to the protein content in the moringa leaves which are 16.67% and 33.33% respectively [21].

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Table 2: Alternative options for halal critical ingredients used in pharmaceuticals and cosmetics (*Contd.)*

No	Themes	Sub-themes/ Supporting Evidence
		Xanthan Gum - An alternative to gelatine. A study shows that xanthan gum is a suitable release-retarding agent in the formulation of halal sustained release gliclazide tablets using the wet granulation technique [22].
		Pectin(Mango peel) - An alternative to gelatine. According to the results of its gelling properties and sensory evaluation, (crude mango peel pectin) CMPP has a high potential to be utilized as low-methoxyl pectin. It also serves as a low-cost gelatin alternate [23].
		Brewer's Rice - The study found that fermented brewer's rice extract increased total flavonoid and total phenolic content while also improving ferric-reducing and antioxidant activities. Furthermore, fermented brewer's rice extract inhibits tyrosinase and elastase more than unfermented extract, by roughly 7- and 57-fold, respectively. Ferulic and kojic acid, two of the most essential components in cosmetic formulations, were also found in fermented brewer's rice extract [24].
2	Animal-based Ingredient	Cyprinus Carpio - An alternative to non-halal animal collagen. The study found that the yield of collagen from carp is around 8.62%, with the characteristic of yellowish-white and a pH of 6.59. Furthermore, the analysis of the carp reveals a fibril structure with chemical interactions dominated by amide groups [25].
		Camel Skin - An alternative to commercial gelatin. According to the study, camel skin was able to produce a yield of 29.1% of gelatin after 2.58 minutes at 71.87 degrees Celsius and pH 5.26. The bloom value of the gelatin from camel skin was 340.4 g. The study also found that the isolated gelatin had a significant proline and glycine content through the amino acid analyzer tool [26].
		Cobia (Rachycentron canadum) skin - An alternative to gelatine. The study indicated that cobia skin gelatin (CG) can binds to fat easily compared to bovine gelatin (BG). However, CG has low moisture retention compared to BG.The lowest concentration of gelling for BG was measured at 1%, while for CG it was 2%. [27].
		(*Contd.)*

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Table 2: Alternative options for halal critical ingredients used in pharmaceuticals and cosmetics (*Contd.)*

No	Themes	Sub-themes/ Supporting Evidence
3	Marine-based ingredient	Seaweed - The antioxidant test revealed that P.pavonica (brown macroalgae) had the highest (1,1-diphenyl-2-picrylhydrazyl) DPPH activity, with an inhibition percentage of 61% . All of the seaweed samples shown high antibiotic activity against E. coli and P. aeruginosa when it was tested for antimicrobial test. The overall antifungal test findings showed that all seaweed samples had moderate antifungal activity against M. gypseum and Fusarium sp [28].
		Microalgae - Alternative to synthetic colourants. The study found that Chlorella vulgaris has the highest carotenoid content. Lutein is found in all six microalgae species, while Pandorina morum contains all three forms of carotenoids: lutein, β-carotene, and β-cryptoxanthin [29].
4	Microbe-based ingredient	Bacteria-producing cellulose - An alternative to commercial cellulose-gelatine. The results of this investigation indicated that bacterial cellulose can be manufactured from Enterobacter sp. M003 with continuous stirring conditions and good carbonaceous materials such as fructose to replace glucose for bacterial cellulose (BC) synthesis [30].
		- An alternative to cellulose. The study discovered that optimum bacterium cellulose was achieved at around 2.28 g/L at 32°C and pH 4 during a 7-day fermentation period. Coconut water is utilised as a medium to ferment bacterial cellulose [31].
		Amillariella Mellea - An alternative to synthetic insulin. This study found that Amillariella mellea, an edible fungus, improved on a dexamethasone (DEX)-induced insulin resistance together with high fat diet(HFD) by regulating lipid metabolism. Our findings suggested that Amillariella mellea extract can be a potential medication in diabetic patient [32].

status unclear[10]. If the substance comes from a different source than synthetic, it will be considered haram[9], although collagen's halal status remains uncertain because it comes from a variety of sources, including humans, animals, and plants. Human and animal collagen is considered haram, while plant collagen is considered halal[11][9]. Aside from that, glycerin's halal status was reviewed because it can be derived from a variety of sources. The supported articles demonstrate that animal-based glycerin requires additional explanation and inquiry into its halal validity when compared to

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Table 3. The testing methods used to test the alternative option for halal critical ingredients

No	Themes	Sub-themes/ Supporting Evidence
1	Morphology Analysis	(Scanning Electron Microscopy) SEM - The current collagen from the market, morphology revealed a heterogeneous fibril form, while microcollagen from cyprinus carpio revealed homogenous particles [25]. - SEM analysis revealed a fine-stranded network structure with thin connective walls in 10% and 13% protein gels made at pH 3.4. The morphology explain the gel's transparency since the homogeneous fine-stranded network allows more light to pass through without scattering [15]. - SEM was used to investigate the morphology of bacterial cellulose. The scanning reveals the compact structure of cellulose generated using the air-drying process[30].
		Field Emission Scanning in Electron Microscopy (FESEM) - FESEM visualizes the bacterial cellulose(BC) that has an entangled structure with mild porous spread through it permitting the impregnation of various compounds into the BC matrix. Hence, the flexibility and stiffness properties of BC are improved [31].
2	Functional groups and Chemical bond Analysis	(Fourier-Transform Infrared) FTIR - This study used FTIR to determine the collagen's functional groups and chemical bonds. The results indicated the presence of both an amide A bond and an amide B position [25]. - The study results showed that both spectra of lard and EVOO seem fairly similar. However, they revealed some differences in peak intensities and the specific wavenumbers at which the highest absorbance were seen in LD(Lard) and EVOO, due to the different nature and composition of both LD and EVOO [33]. - The study's results revealed that the two samples have identical FTIR peaks, with the exception of some areas where bacterial cellulose did not produce a strong peak like microcrystalline cellulose due to its compacted structure. However, the study proves that Enterobacter sp. M003 produces an authentic bacterial cellulose [30].
		(*Contd.)*

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Table 3. The testing methods used to test the alternative option for halal critical ingredients (*Contd.)*

No	Themes	Sub-themes/ Supporting Evidence
		UV-Vis spectrophotometer - In this study, UV absorption was assessed on collagen samples isolated from carp scale waste using a UV-visible spectrophotometer. The results show that the benzene causes a bathochromic shift, particularly in the K band (204nm), which shifts to a wavelength of 268nm. The shift results not just from benzene aromatics, but also from alkyl substituents and functional groups. It denotes the existence of carboxyl and hydroxyl groups [25].
		(Gas chromatography-mass spectrometry) GCMS - The volatile components of the triplicate A.malaccensis leaf extract samples were determined using a gas chromatography equipment. The findings revealed that different types of extracts produced varying numbers of peaks and compounds [14].
3	Particle Size Analysis	(Particle Size Analyzer)PSA - PSA was used to measure the size and distribution of micro-collagen particles.The obtained results ranged from 668 nm (d10 < 10%) to 1581 nm (d90 < 90%). The micro-collagen particle size with the highest distribution intensity was 1146 nm [25].
4	Activity Analysis	2, 2-diphenyl-2-picrylhydrazyl (DPPH) Scavenging assay - The sample with high antioxidant activity has a high amount of Gum Arabic (GA). Recent studies have also shown that GA has antioxidant qualities because it plays a role in lipid metabolism and improves kidney failure and cardiovascular treatment [13]. - This test was designed to investigate the antioxidant effects of fermented brewer's rice extracts. The results revealed that fermented samples had higher biological components and antioxidant activity than their unfermented counterparts which is favourably connected with total phenolic content [24].
		Quantitative reverse transcription polymerase chain reaction (qRT-PCR) - The results showed that myricitrin therapy prevented alloxan-induced gene expression degradation or diabetes development. Among the altered genes, IκBα, STMN1b, and IL1b were associated with toll-like receptor pathways. The toll-like receptor detects β-cell death. Furthermore, inhibiting toll-like receptors can reduce β-cell mortality in diabetes [19].

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Table 3. The testing methods used to test the alternative option for halal critical ingredients (*Contd.)*

No	Themes	Sub-themes/ Supporting Evidence
		Ferric reducing antioxidant power (FRAP) Assay - This experiment was designed to investigate the antioxidant effects of fermented brewer's rice extracts. The results revealed that fermented samples had higher biological components and antioxidant activity than their unfermented counterparts which is favourably connected with total phenolic content [7].
5	Texture profile analysis	Texture Analyzer - The study results revealed that Gum Arabic had decreased hardness, cohesiveness, and springiness. Furthermore, it has a lower gumminsess value than gelatine, therefore it does not require a lot of energy to break down the pastille [13]. - The study found that the Bloom value (gelatin strength) of camel skin gelatin was 340 ± 0.5 g. This number is comparable to gelatin from bovine which was higher (267g) and almost similar amount to porcine source gelatin(350g) [26].
6	Component/ Content Analysis	HPLC System - HPLC tool was used to quantify the total carotenoid content. The content of chlorophyll for both P. pavonica and C. lentillifera is similar .It was found that the chlorophyll composition in K. striatum is 4.6 µg/g DW, G. tikvahiae at 2.9 µg/g DW, and E. denticulatum at 3.0 µg/g DW so it showed that the chlorophyll content in these 3 compound are lower compared to P. pavonica and C. lentillifera [28]. - Protein amino acid analysis was performed using an HPLC system. Both asparagine and aspartic acid, as well as glutamine and glutamic acid, were considerably greater in ASE-PPI (pea protein isolate extracted via ammonium sulphate precipitation technique) than in AE-PPI [15]. - The HPLC technique found three significant carotenoid peaks, including lutein, β-cryptoxanthin, and β-carotene.Lutein and β-carotene concentrations were highest in C. fusca (63.39 ±5.99 µg/g DW) and C.vulgaris (18.42 ± 5.31 µg/g DW), respectively [29]. - Amino acids were analysed using an HPLC technique. Camel skin gelatin has high glycine, proline, and lysine levels, similar to bovine and porcine gelatins. Camel skin gelatin contained more lysine than porcine or bovine gelatin [26].

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Table 3. The testing methods used to test the alternative option for halal critical ingredients (*Contd.)*

No	Themes	Sub-themes/ Supporting Evidence
		Chemometrics - Using the score plot projection, cream containing lard, EVOO, and commercial samples are highly separated, indicating that PCA (Principal Component Analysis) can classify them. Based on this profile, commercial samples (region B) do not contain lard in their formulation [33]. - Fuzzy Autocatalytic Set (FACS) is a novel chemometrics method where it discovered the FTIR spectra peaks of bovine, porcine, and fish gelatins.The method was successful because prominent peaks were able to observed and they are differ among the different types of gelatins. Hence, this method provides a warrant for halal authentication [34].
		LC-MS/MS Analysis - The research revealed that the active content which was myricitrin, higher than the other compounds which are myricetin-3-rutinoside,,myricetin-3-O-pentoside, and myricetin. It remained steady throughout tree age samples [19].
		Amino acid analyser - According to the findings, the total amino acid content in (Bovine Gelatin) BG and (Cobia Gelatin) CG is 99.51% and 86.65%, respectively [27].
		Modified Quartz Crystal Microbalance (QCM) sensor method - The modified QCM sensor produced a satisfactory frequency response for distinguishing bovine and porcine gelatin. The measurements produced negative frequency shifts for bovine gelatin and positive frequency shifts for porcine gelatin, indicating that bovine gelatin is halal and porcine gelatin is non-halal [35].

alternative sources, such as plant-based, microbial-based, or even propylene gas. According to this review, there are several alternate options for insulin as listed in Table 3. Three articles show that *Bolanthus spergulifolius*, *Amillariella mellea*, and *Acer truncatum* leaves, respectively, provide an excellent replacement for insulin as a halal critical ingredient. A study found that Acer truncatum leaves containing myricitrin can repair gene expression associated with diabetes by interfering with toll-like receptor pathways, improving glucose absorption, and relieving hyperglycemia levels[19]. On the other hand, a study found that *Bolanthus spergulifolius* had a similar impact as insulin when the substance was treated on adipocytes and produced a positive effect in

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terms of gene expression[18]. Furthermore, Amillariella Mellea dramatically lowered fasting blood glucose levels, lowering glucose intolerance and insulin resistance via raising the expression of two important lipases[31]. As a result, all these drugs are effective alternatives to insulin. Finally, gelatine also was found to have many alternatives according to this review where 6 articles suggested an alternative option such as gum arabic, xanthan gum, pea protein isolate, pectin, camel skin, and cobia skin respectively. All these alternative options possess similar efficacy and features as gelatin. For example, Gum Arabic is an important hydrocolloid that can be used in pastille production and acts as a stabilizer and fat emulsifier[13] on the other hand xanthan gum possesses the best release-retarding agent so it can used in various formulations of drug for a sustained-release effect[22]. These effects are also performed by gelatin. Since all the alternative options can be obtained naturally it will produce a much more cost-effective halal product and at the same time, it maintains the quality of the product. Hence, these substances can be confidently used by the manufacturers to produce a halal product. This review also covers the testing methods that were used to analyze the alternative halal critical ingredients so that they can be used for future product formulations which are shown in Table 4. A total of 24 articles used different types of testing methods to characterize the alternative halal critical ingredient. Scanning Electron Microscopy (SEM) is one of the common testing methods used to analyze the morphology of the compound. 5 articles used SEM as one of their testing methods to visualize the compound. Besides that, FTIR was used commonly to study the functional groups and chemical bonds of a compound. 8 articles used FTIR to analyze their compound. Furthermore, the researchers

Table 4: The testing methods used to test the alternative option for halal critical ingredients

No	Themes	Sub-themes/ Supportive evidence
1	Morphology Analysis	**(Scanning Electron Microscopy) SEM** – The current collagen from the market, morphology revealed a heterogeneous fibril form, while microcollagen from cyprinuscarpiorevealed homogenous particles [25]. – SEM analysis revealed a fine-stranded network structure with thin connective walls in 10% and 13% protein gels made at pH 3.4. The morphology explains the gel's transparency since the homogeneous fine-stranded network allows more light to pass through without scattering [15]. – SEM was used to investigate the morphology of bacterial cellulose. The scanning reveals the compact structure of cellulose generated using the air-drying process [30]. **Field Emission Scanning in Electron Microscopy (FESEM)** – FESEM visualizes the bacterial cellulose (BC) that has an entangled structure with mild porous spread through it permitting the impregnation of various compounds into the BC matrix. Hence, the flexibility and stiffness properties of BC are improved [31].

(*Contd.*)

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Table 4: The testing methods used to test the alternative option for halal critical ingredients (*Contd.*)

No	Themes	Sub-themes/ Supportive evidence
2	Functional groups and Chemical bond Analysis	**(Fourier-Transform Infrared) FTIR** – This study used FTIR to determine the collagen's functional groups and chemical bonds. The results indicated the presence of both an amide A bond and an amide B position [25]. – The study results showed that both spectra of lard and EVOO seem fairly similar. However, they revealed some differences in peak intensities and the specific wavenumbers at which the highest absorbance were seen in LD(Lard) and EVOO, due to the different nature and composition of both LD and EVOO [33]. – The study's results revealed that the two samples have identical FTIR peaks, with the exception of some areas where bacterial cellulose did not produce a strong peak like microcrystalline cellulose due to its compacted structure. However, the study proves that Enterobacter sp. M003 produces an authentic bacterial cellulose [30]. **UV-Vis spectrophotometer** – In this study, UV absorption was assessed on collagen samples isolated from carp scale waste using a UV-visible spectrophotometer. The results show that the benzene causes a bathochromic shift, particularly in the K band (204nm), which shifts to a wavelength of 268nm. The shift results not just from benzene aromatics, but also from alkyl substituents and functional groups. It denotes the existence of carboxyl and hydroxyl groups [25]. **(Gas chromatography-mass spectrometry) GCMS** – The volatile components of the triplicate A.malaccensis leaf extract samples were determined using a gas chromatography equipment. The findings revealed that different types of extracts produced varying numbers of peaks and compounds [14].

(*Contd.*)

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Table 4: The testing methods used to test the alternative option for halal critical ingredients (*Contd.*)

No	Themes	Sub-themes/ Supportive evidence
3	Particle Size Analysis	**(Particle Size Analyzer)PSA** – PSA was used to measure the size and distribution of micro-collagen particles.The obtained results ranged from 668 nm (d10 < 10%) to 1581 nm (d90 < 90%). The micro-collagen particle size with the highest distribution intensity was 1146 nm [25].
4	Activity Analysis	**2, 2-diphenyl-2-picrylhydrazyl (DPPH) Scavenging assay** – The sample with high antioxidant activity has a high amount of Gum Arabic (GA). Recent studies have also shown that GA has antioxidant qualities because it plays a role in lipid metabolism and improves kidney failure and cardiovascular treatment [13]. – This test was designed to investigate the antioxidant effects of fermented brewer's rice extracts. The results revealed that fermented samples had higher biological components and antioxidant activity than their unfermented counterparts which is favourably connected with total phenolic content [24]. **Quantitative reverse transcription polymerase chain reaction (qRT-PCR)** – The results showed that myricitrin therapy prevented alloxan-induced gene expression degradation or diabetes development. Among the altered genes, IκBα, STMN1b, and IL1b were associated with toll-like receptor pathways. The toll-like receptor detects β-cell death. Furthermore, inhibiting toll-like receptors can reduce β-cell mortality in diabetes [19]. **Ferric reducing antioxidant power (FRAP) Assay** – This experiment was designed to investigate the antioxidant effects of fermented brewer's rice extracts. The results revealed that fermented samples had higher biological components and antioxidant activity than their unfermented counterparts which is favourably connected with total phenolic content [7].

(*Contd.*)

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Table 4: The testing methods used to test the alternative option for halal critical ingredients (*Contd.*)

No	Themes	Sub-themes/ Supportive evidence
5	Texture profile analysis	**Texture Analyzer** – The study results revealed that Gum Arabic had decreased hardness, cohesiveness, and springiness. Furthermore, it has a lower gumminsess value than gelatine, therefore it does not require a lot of energy to break down the pastille [13]. – The study found that the Bloom value (gelatin strength) of camel skin gelatin was 340 ± 0.5 g. This number is comparable to gelatin from bovine which was higher (267g) and almost similar amount to porcine source gelatin(350g) [26].
6	Component/ Content Analysis	**HPLC System** – HPLC tool was used to quantify the total carotenoid content. The content of chlorophyll for both P. pavonica and C. lentillifera is similar. It was found that the chlorophyll composition in K. striatum is 4.6 µg/g DW, G. tikvahiae at 2.9 µg/g DW, and E. denticulatum at 3.0 µg/g DW so it showed that the chlorophyll content in these 3 compound are lower compared to P. pavonica and C. lentillifera [28]. – Protein amino acid analysis was performed using an HPLC system. Both asparagine and aspartic acid, as well as glutamine and glutamic acid, were considerably greater in ASE-PPI (pea protein isolate extracted via ammonium sulphate precipitation technique) than in AE-PPI [15]. – The HPLC technique found three significant carotenoid peaks, including lutein, β-cryptoxanthin, and β-carotene.Lutein and β-carotene concentrations were highest in C. fusca (63.39 ±5.99 µg/g DW) and C.vulgaris (18.42 ± 5.31 µg/g DW), respectively [29]. – Amino acids were analysed using an HPLC technique. Camel skin gelatin has high glycine, proline, and lysine levels, similar to bovine and porcine gelatins. Camel skin gelatin contained more lysine than porcine or bovine gelatin [26]. **Chemometrics** – Using the score plot projection, cream containing lard, EVOO, and commercial samples are highly separated, indicating that PCA (Principal Component Analysis) can classify them. Based on this profile, commercial samples (region B) do not contain lard in their formulation [33].

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Table 4: The testing methods used to test the alternative option for halal critical ingredients (*Contd.*)

No	Themes	Sub-themes/ Supportive evidence
		– Fuzzy Autocatalytic Set (FACS) is a novel chemometrics method where it discovered the FTIR spectra peaks of bovine, porcine, and fish gelatins.The method was successful because prominent peaks were able to observed and they are differ among the different types of gelatins. Hence, this method provides a warrant for halal authentication [34]. **LC-MS/MS Analysis** – The research revealed that the active content which was myricitrin, higher than the other compounds which are myricetin-3-rutinoside,,myricetin-3-O-pentoside, and myricetin. It remained steady throughout tree age samples [19]. **Amino acid analyser** – According to the findings, the total amino acid content in (Bovine Gelatin) BG and (Cobia Gelatin) CG is 99.51% and 86.65%, respectively [27]. **Modified Quartz Crystal Microbalance (QCM) sensor method** – The modified QCM sensor produced a satisfactory frequency response for distinguishing bovine and porcine gelatin. The measurements produced negative frequency shifts for bovine gelatin and positive frequency shifts for porcine gelatin, indicating that bovine gelatin is halal and porcine gelatin is non-halal [35].

commonly used the DPPH Scavenging assay to study the activity of a compound, especially the antioxidant properties. 3 articles that study the antioxidant properties used this DPPH assay. Other methods were also used to analyze the activity such as qRT-PCR and FRAP assay. However, these methods were only used in a maximum of 1 article respectively. The texture of a compound was analyzed using a texture analyzer commonly. To support this statement, 3 articles had used this method. On the other hand, HPLC systems and chemometrics are commonly used to study the content or composition of a substance. 5 and 3 articles used this method to figure out the content of their study compound respectively. In conclusion, the testing methods that were discussed above are commonly used in the research setting. However, there are other methods too such as the methods that were listed in Table 3. This review will serve as a guide for researchers on the different types of testing methods that can be used for the analysis of a product or substance.

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Conclusion

In conclusion, this study suggested alternative options for halal critical ingredients in halal pharmaceuticals and cosmetics. All the research objectives and the aim of this study were achieved. Hence, this studyconcludes that among the articles that were collected for this review study, insulin, and gelatine are the most commonly studied. Their alternative options whether it's from plant sources, marine sources, or microbe-based ingredients are tested extensively to assess for their desired effect or activity. The testing methods proved that the alternative options are much better than the critical ingredients in terms of their texture, morphology, activity, composition, and even the cost of synthesis.

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A Systematic Literature Review of Hemicellulose-Based Hydrogels for Drug Delivery System- A Review

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Abstract

Development of hemicellulose-based hydrogels have gained many interests from researchers in recent years because of their excellent biocompatibility, biodegradability, economically, and non-toxic. Hemicellulose-based hydrogels possessed exclusive properties such as tuneable swelling behaviour and stimuli-responsiveness which have advantages in the preparation of potential hydrogels for drug delivery applications. This study was conducted by a systematic review process guided by PRISMA protocol, involving 20 related studies retrieved from Science Direct and PubMed, and data extraction and analysis were done by thoroughly review all the included articles. Three main themes were raised and discussed mainly focusing on the hydrogel synthesis, properties and drug delivery applications. The findings of this study had shown that hemicellulose-based hydrogels have great performance, functionality, and proven to be a promising drug carrier for controlled and sustained-release drug delivery.

Keywords: Hemicellulose; Hydrogel; Drug delivery

Introduction

In a recent study, hydrogels have been defined as two- or multi- component system that is made up of three-dimensional (3D) networks which are formed by crosslinking the polymer networks. It was found that hydrogel possessed a unique property where they have the ability to absorb and retain a large amount of water in the interstitial spaces between the networks, but unable to dissolve in the surrounding medium (1-3). Hydrogels that contain interactive functional groups along their main polymer chains are commonly known as stimuli-responsive hydrogels. The stimuli-responsive hydrogels are hydrogels which are sensitive to specific environment stimulus changes and exhibit responses by changing their shape and size, changes in its optical, wettability, electric signal and mechanical properties (4,5).

Polymers are resourceful materials composed of repeating structural units forming a macromolecule. There are three main classes of polymers based on their origin which are natural polymers, semisynthetic polymers (or also known as hybrid polymers), and synthetic polymers. Natural polymers are obtained from the natural sources or origins such as plants, animals, and microorganisms. Natural polymers are economically, readily available, potentially biocompatible, and biodegradable in comparison to synthetic and semisynthetic polymers due to their origin (6).

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Polysaccharides are one of the biopolymer classes use in the development of hydrogel preparations due to their useful biocompatibility, biostability and biodegradability as natural polymers (7) which make them harmless and suitable for several biomedical applications such as cell or drug delivery, gene delivery, cell culture, regenerative medicine and tissue engineering.

Hemicellulose is the second most abundant natural polysaccharides after cellulose, that is extensively exists as the component of plant cell walls, and constitute about 20 to 30 percent of the total weight of lignocellulosic biomass. However, unlike cellulose which comprised of 7,000 to 15,000 sugar units per polymer, hemicellulose has shorter chains of 500 to 3,000 sugar units (8).Development of hemicellulose-based hydrogels have gained many interests from researchers in recent years not only because they are from renewable biopolymer resources and cost-effective, but also because hemicellulose-based hydrogels have significant physicochemical properties, including biocompatibility, biodegradability, non-toxic and anti-cancer effect. These exclusive properties of hemicellulose have advantages in the preparation of potential hydrogels for drug delivery applications.

Methodology

Prisma

The reviewers decided to adopt a publication standard called PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-analyses) statement that was first published in 2009. PRISMA is a reporting guideline which aimed to address the poor reporting of systematic reviews by guiding the reviewers to write an accurate, complete, and transparent reports (9). This standard suggests the inclusion of several key points as stated in the PRISMA checklist such as title, abstract, methods, results, discussion and funding sections, with details of content that should be included in each section such as search strategy process, review process, and document selection process. The suitability of PRISMA to be use in this systematic review is because of these benefits: (1) it clearly defines the research questions; (2) it able to identify the inclusion and exclusion criteria, and (3) it attempts to assess and examine the largest amount of available and relevant scientific literature within a specific time period (10).

Resources

Two leading academic research databases were used for this review, which are Science Direct and PubMed. Both databases are considered the leading trusted resources for citations. Both databases published peer-reviewed articles and can be accessed for free. Science Direct is Elsevier's platform which serves as a gateway to millions of academic articles. These two databases are chosen because of their prominence, which is important to ensure the quality of the articles reviewed in this study.

Systematic review process

Identification

Identification is the first phase in the systematic review process of this study, performed in March 30th, 2021. The process involved identification of keywords for information searching purposes. Researchers were using several relevant information sources such as the dictionaries, encyclopaedias, thesaurus and keywords suggested by Science Direct and PubMed for keywords synonyms, possible related terms, and other variations to the term 'drug delivery' were used. Therefore, in this process, the following search items were used for the documents search (see Table 1).

Screening (Inclusion and Exclusion Criteria)

Screening is a phase where the reviewers will determine the criteria of articles are to be included or excluded with the

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Table 1: Keywords and search items for documents retrieval

Databases	Keywords and search items used for systematic searching	
Science Direct, PubMed	(Hemicellulose hydrogel)	AND (Drug delivery OR drug release OR drug carrier OR deliver drug OR release drug)

Table 2: The inclusion and exclusion criteria

Criteria	Inclusion	Exclusion
Document type	Journals (research articles)	Journals (review articles), books, book chapters, conference proceedings
Timeline	Between 2010 to 2021	< 2010
Language	English	Non-English

assistance of the specific databases. In the screening process, inclusion and exclusion criteria were decided to search for suitable articles to be included on the systematic review process. Before the screening process was carried out, duplicate documents were first removed from the retrieved articles. The inclusion and exclusion criteria are as shown in (Table 2).

Eligibility

The third phase is the eligibility phase, which is a manual process of including or excluding the full-text articles according to the reviewers' specific criteria. The articles retrieved were thoroughly reviewed in this process, where any articles that did not meet the criteria were excluded from the study. The articles were screened manually for literature focusing on hemicellulose-based hydrogels for drug delivery application and criteria from the earlier screening processes (inclusion and exclusion criteria). Any papers which do not involve the study on drug delivery application of hemicellulose-based hydrogels were excluded. Additionally, articles that inaccessible were also excluded from the eligibility process.

Assessment of quality

For the assessment of quality of the included papers, this process was done to evaluate the quality of papers to be reviewed and to prevent the risk of bias in reporting. In this review, quality assessment was done by adopting the method suggested by (11), where the remaining included articles obtained in the last process of systematic review were presented, evaluated and ranked by the experts of the topic into three different quality categories namely as high, moderate, and low. In order to maintain good quality reporting, only articles that are ranked in high and moderate categories are to be reviewed. To evaluate the article's rank of quality, experts should focus on the methodology and the finding outcomes of each individual article. Next, authors must come to a mutual agreement that the rank of quality for article must be at least at a moderate level in order to be included in the review. Any disagreement arises should be thoroughly discussed in a professional manner among the authors before deciding on the inclusion or exclusion of the remaining articles for the review.

Data Extraction and Analysis

The remaining articles left from the eligibility process included in this study were evaluated, reviewed, and analysed. Before reviewing the included articles, a spreadsheet form was created to gather the general data compiles the authors, title, journal, publication year, and summary of findings. Data of each article was extracted by reading through the abstracts first, and then the full-text which include an in-depth reading of the whole article to identify and extract the available data.

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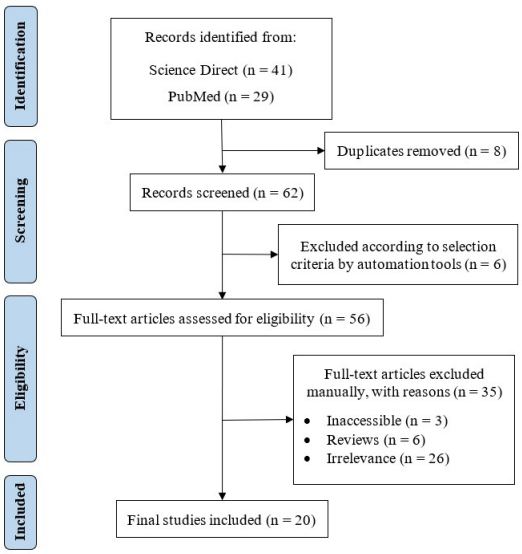

Figure 1: Flow diagram following the PRISMA guidelines, showing the identification, screening, eligibility, and final studies included. 20 papers were included (search performed on March 30th, 2021)

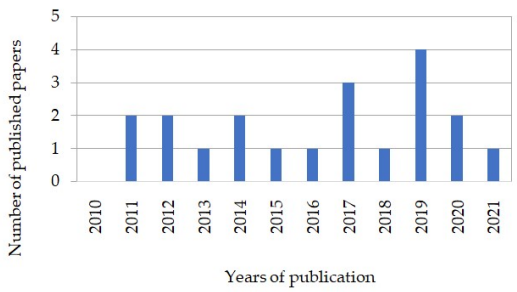

Figure 2: Distribution of published papers per year

A thematic analysis of available data was performed for this study to discuss all the data extracted into different themes and sub-themes. In this process, the extracted data were transformed by authors into useful data and guided by the identification of the concepts, ideas and themes which connect and relate all the available data from the included articles. The final themes discussed are: (1) hydrogels synthesis; (2) hydrogels properties, and (3) drug delivery applications. The themes and sub-themes were developed together with the co-authors by corresponding author. Discussion and re-evaluation of the themes and sub-themes were conducted on ongoing basis. Any disagreements or inconsistencies were resolved in a proper and professional manner. No meta-analysis was done in this review because of the heterogeneity of studies.

Results and Discussion

Findings

Based on the advanced systematic search on the two databases, a total of 70 studies were obtained, where 41 studies were found in Science Direct and 29 studies were found in PubMed using the identified keywords search string. A total of eight studies traced as duplicate articles were excluded first before the screening process, which resulted to 62 studies left. Six studies were excluded automatically by the databases after the inclusion and exclusion criteria filters were applied. Out of 56 studies left, a total of 35 studies were manually excluded from the eligibility process for various reasons, where three studies were inaccessible, six studies were review articles, and 26 studies were irrelevant to this study. Thus, only 20 studies were included for this study (see Figure 1).

Number of Papers Collected by Selection of Keywords

Several keywords were generated in order to refine the systematic searching when collecting the studies with regard to the research question. Table 3 shows the number of papers collected by selection of keywords from each database. Important to note that eight out of the 29 papers identified from PubMed were similar papers retrieved from Science Direct, and were excluded as duplicate papers.

Number of publication by year

The publication year of articles reviewed as described in the inclusion

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criteria were ranging from year 2010 to 2021. Figure 2 shows the distribution of published papers per years. There were no paper prior to 2011, and only one paper published in the first-half of this year period that is related the topic of this study. As shown in Figure 2, the year 2019 have the most published papers regarding hemicellulose-based hydrogels for drug delivery system.

Quality assessment

All the remaining 20 included articles were ranked to be of good quality by the experts of the topic, with 12 of them ranked as high quality, and eight of them ranked as moderate quality. The 20 included articles were thoroughly evaluated and ranked by the experts by focusing on the methodology and the finding outcomes that are related to the topic of this review. As per agreement with the authors, only articles of high and moderate quality were allowed to be included in the review. Since, all the articles were ranked moderate and above, thus all the remaining 20 included articles were eligible for the review.

Journal analysis

In this section, a classification of journals has been made to analyse what type of publications correlates with our topics. Four main categories and other two subcategories from SCImago Journal & Country Rank (SJR) were used to group the journals. The main categories include: (1) material, journals of materials chemistry/material related topics; (2) chemistry, journals focused on the organic chemistry/biochemistry topics; (3) pharmaceuticals, journals focus on the pharmaceutics/biopharmaceutics topics, and (4) multidisciplinary, journals that allow for multiples topics. In addition, the four combined subcategories include: (1) chemistry/material, and (2) material/pharmaceuticals.

Figure 3 shows the distribution of all 11 journals with the number of papers published from each respective journal

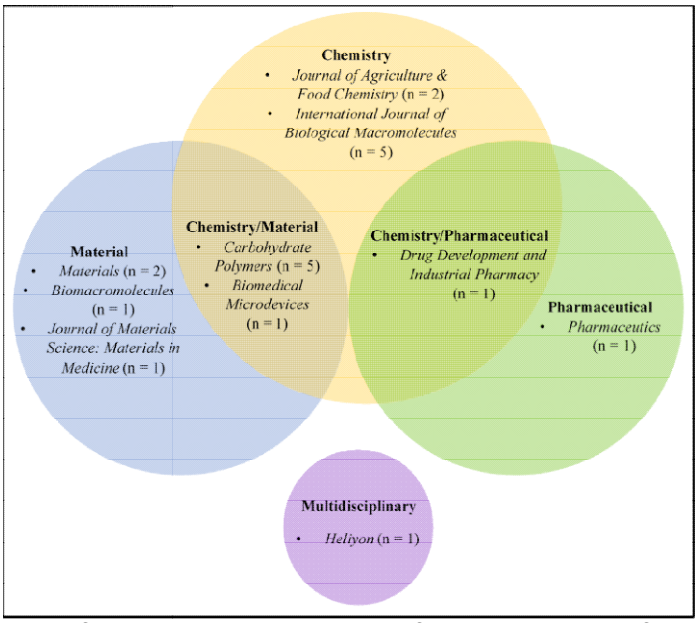

Figure 3: Distribution of journals with the number of papers published from each respective journal category

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category. Journals under Chemistry category contains the highest number of papers retrieved where five out of 20 papers included were from the “International Journal of Biological Macromolecules”. This is because the journal focused on the studies of molecular structure and properties of natural macromolecules, and this include macromolecular carbohydrates which is closely related to our topics. The second highest number of papers retrieved were from the chemistry/material subcategory where “Carbohydrate Polymers” published five out 20 papers included. This is expected to be as this journal covers the study and exploitation of polysaccharides on their potential applications in several areas, where one of them is on drug delivery application. Thus, papers retrieved from this journal correlates best to our topics. The remaining categories and sub-category contain fewer papers, where only one or at most 2 papers were published from them.

Hydrogel synthesis

Materials

Hemicellulose is one of the potential choices of biopolymer material for the preparation of natural hydrogels. This is mainly due to its great advantages which include biocompatibility, biodegradability, economical and most importantly is non-toxic that had been described throughout many studies. Hemicellulose extract in most studies was reported to be extracted from various different plant sources such as bagasse, bamboo, beech wood, hardwood pulp, maize bran, oat spelt, psyllium husk, quince, tamarind seed powder, and wheat straw. Among the many types of hemicelluloses, xylan-type hemicelluloses are the most commonly used as mentioned in 14 out of 20 studies as shown in Table 4, this is probably because xylan is the major hemicellulosic component of plants, and constitute about 20-35% of the biomass, thus making xylan the most abundantly found component from agriculture, forestry, pulp, and paper industries.

Several monomers have been studied in preparation of hemicellulose derivatives-based hydrogels. As shown in Table 4, these monomers include acrylic acid (AA), acrylamide (AM), maleic anhydride (MA), and others. Among all those monomers, acrylic acid is the most used monomer in hydrogel preparation appearing in a total of 7 studies. AA is considered as an important monomer which is widely studied and used for the development of functional hydrogels because it was proven to be effective in forming hydrogel in many studies. Moreover, incorporation of synthetic monomer like AA into the hemicellulose-based hydrogels had shown great improvement in the hydrogel properties, in terms of their water adsorption, swelling capacity, and stimuli responsiveness. Thus, these improvements allow for the hemicellulose-based hydrogels to enhance their uses in drug delivery applications.

Methods of preparation

The preparation of hemicellulose-based hydrogels involved the crosslinking procedure of hemicellulose polymers, with or without presence of other polymers and monomers. In this review, all 20 studies had use different chemical crosslinking approaches in forming the hemicellulose-based hydrogels, where free radical copolymerization was the most commonly used chemical crosslinking method as mentioned in 10 out of 20 studies. Followed with, solution polymerization with 4 studies, and enzymatic reaction with 3 studies. As for the other remaining 3 studies, each had approached with different crosslinking method such as suspension polymerization, reactive extrusion process, and one-pot reaction. Table 5 shows the summary of the crosslinking methods of the hemicellulose-based hydrogels analysed from all the included studies.

Crosslinking by free radical copolymerization

In this review, the most extensively studied crosslinking method for hemicellulose-based hydrogels preparation

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is free radical copolymerization which appeared in 10 studies. This free radical copolymerization method is gaining much interest in preparing hydrogels for bioapplications, including for drug delivery applications, mainly because of its efficiency in the rapid formation of the hydrogel, even if it were done under mild conditions. This method usually involves the grafting of monomer onto the backbone of activated polymer chains, which results to branching and crosslinking between the polymers and monomers, thus forming the hydrogels. Generally, the polymer chains can be activated by the action of various chemical initiators and high-energy irradiations (12), and as to date many initiation systems were introduced for free radical copolymerization method. For instance, there were 3 studies performed by Gao et al., Kong et al., and Yang et al. using photoinitiator such as 2,2-Dimethoxy-2-phenylacetophenone (DMPA) to generate the free radicals when exposed under UV irradiations. DMPA is considered as a highly efficient and most extensively used photoinitiator for UV polymerization, due to their stability. While in another study by Sun et al., they performed the redox initiation system where they successfully grafted the monomer, acrylic acid (AA) onto the backbone of arabinoxylan-type hemicellulose, by using potassium persulfate and anhydrous sodium sulphite as the redox initiator. The polymerization in this study was activated via the redox polymerization by the reduction-oxidation reaction occurred between the oxidizing agent (potassium persulfate) and reducing agent (anhydrous sodium sulphite). Similar crosslinking method using redox initiation system was performed in another 2 studies by Chen et al. and Peng et al.. Meanwhile, there were also other initiator systems mentioned in this review, where 2 studies performed KPS initiator system using potassium persulfate (KPS) as the free radical initiator which activated at temperature of 70 °C, and 1 study each performed free radical polymerization by using azobisisobutyronitrile (AIBN) initiator and ammonium persulfate (APS) initiator, respectively, to generate the radicals for the free radical polymerization.

Hydrogel properties

Porous structure

The morphological characterization of hydrogels is one of the important parameters in hydrogel preparation. This is because morphological structure of hydrogels is differed from changes in the hydrogel's composition to the preparation method, or by any interactions occurred within the polymer matrix (13). As mentioned, slight changes in hydrogel properties, including the structure of hydrogel matrix may influenced the applications of hydrogels. In this review, the morphological characterization of hemicellulose-based hydrogels was analysed by using the scanning electron microscope (SEM) as mentioned in most studies. Among them, 3 studies had presented the freeze-dried hemicellulose-based hydrogels possessed a macroporous structure and the other 4 studies had described the freeze-dried hemicellulose-based hydrogels possessed honeycomb-like porous structure. Meanwhile, 2 studies by Chen et al. and Sun et al., mentioned the resulting freeze-dried hemicellulose-based hydrogels presented with both macroporous and honeycomb-like structure. In overall findings, among all the studies mentioned above, the porous structure of hydrogel matrix can be increased with increasing of crosslinking density, as more open and looser pore network structures were formed. However, with continuous increasing of crosslinking density, it may also lead to uneven crosslinking density which resulted to formation of uneven network structure or cracklike structure. Moreover, the importance of knowing the porous structure of hydrogels was mentioned by this author, where macroporous structure of hydrogels resulted to increase amount of water absorption capacity into the hydrogel matrix, where it is generally known that the

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increasing water absorption capacity is closely related to the increasing swelling capacity of hydrogels, thus may impact the applications of the resulting hemicellulose-based hydrogels for drug delivery system.

Swelling capacity

Swelling ratio (SR) is another important characterization parameter in every hydrogel preparation as it represents the capacity of water being absorbed within the hydrogel matrix and is a function of water retention for the hydrogels. The importance of studying the SR of hydrogels is because it demonstrated the swelling capacity of the resulting hydrogels, and it is considered as the most important property which influenced the applications of hydrogels for drug delivery system. In overall findings of all the studies reviewed, most authors described the SR of hemicellulose-based hydrogels were shown to be influenced with the changes to their network structure, where an increase in SR can be seen in a more expanded or less dense network structure of the hydrogels. Whereas, in a less expanded or denser network will resulted to decrease in the SR. Moreover, the authors also reported that these changes in the hydrogel network structure were mainly due to the effect of changes in hydrogel composition, where 5 studies mentioned the effect of monomer contents and 6 studies mentioned the effect of crosslinker contents had resulted to differed network structure formed during the preparation of hydrogels. There is also 1 study performed by Chang et al. where they introduced pore-forming agents to directly alter the network structure of the hydrogels in order to develop their most desired hydrogels. The summary of these changes and their effects on the hydrogels performed in the studies mentioned above are shown in Table 6. Conclusively, the importance of discovering all the possibilities that could change the structure of hydrogels for this review may be useful in order to guide the future researchers in developing the most-suited and functional hydrogels for drug delivery system.

Effect of monomer

The use of monomer in newer hemicellulose-based hydrogels preparation is significant as it able to improve the properties of pure natural hydrogels, include enhancing their swelling capacity. The effect of monomer content of swelling capacity was reported in 2 studies to have an inverse relationship, where increasing in monomer contents will resulted to a denser network and smaller pore size, thus less water absorption into the hydrogels. Hence, resulted to decrease in SR of hydrogels. However, a contradict result was found in 2 studies, where increasing in monomer contents was resulted to hydrogels had a better water absorbency due increase in monomer molecules to copolymerized with hemicellulose, then formed a better hydrogels network structure and promoted hydrophilicity of the resulting hydrogels. Thus, increase the SR of hydrogels. The reason of these two contradictory results was likely because of there is an optimum value of monomer to hemicellulose weight ratio which serve as the cut-off level whether further increasing of the monomer contents would either improve the SR or reduce the SR. It was found that when the monomer contents were increased to the optimum value, the SR of hydrogels increased. However, when the monomer contents were increased beyond the optimum value, the SR of hydrogels decreased as demonstrated in 1 study by Sun et al.

Effect of crosslinker

Crosslinkers have a significant presence in the hydrogels because they are able to prevent dissolution of the hydrophilic polymer chains in an aqueous environment. The effect of crosslinkers content on swelling capacity was shown to have an inverse relationship where increasing the weight ratios of crosslinkers to hemicellulose, will resulted to the decrease in swelling capacity. This is because increasing the crosslinkers concentration during the preparation of hydrogel, will further increased the degree of crosslinking, and more crosslinking points in

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the hydrogel network structure were produced. This causes the pores became denser, and their diameters became smaller, thus less free spaces among the networks can retain water, which consequently lead to decrease in swelling capacity as demonstrated in 6 studies. The effect of crosslinkers on swelling capacity is important because it was later found that moderate crosslinking density is the principal for drug release and degradation of hydrogels.

Effect of Pore-forming Agents

Apart from altering monomer and crosslinker contents, addition of pore-forming agents into the hydrogel preparation may also change the network structure of hydrogels by modifying the pore size. A study by Chang et al. had shown that different pore-forming agents had different influence on the swelling behaviour of CMX-based hydrogels, where the six different agents were: polyvinylpyrrolidone (PVP) K30, polyethylene glycol (PEG) 2000, carbamide, NaCl, $CaCO^{-3}$, and $NaHCO_3$. Based on results, hydrogels without any pore-forming agents did not reach swelling behaviour within 24 h. In contrast, hydrogels with $CaCO^{-3}$, and $NaHCO_3$ which had the largest pore size, reached swelling equilibrium after 6 h. Meanwhile, hydrogels with PEG 2000 had smaller pore size in comparison to hydrogels without any pore-forming agents, and could not reach the swelling equilibrium after 24 h. Thus, the swelling behaviour of hydrogels is related to the size of their network structure pore. Hydrogels with macroporous structure are more favourable to the diffusion of water molecules into the hydrogels network. Therefore, these macroporous hydrogels had better and faster swelling capability (20).

Stimuli-responsive

In this part, we have reviewed most studies of hemicellulose-based hydrogels on their stimuli-responsive behaviour, and overall findings had demonstrated hemicellulose-based hydrogels have an excellent stimuli responsiveness towards many external stimuli. Among them, 3 studies described the hemicellulose-based hydrogels to show response with changes in temperature of the surrounding medium 10 studies showed response with changes in pH of the surrounding medium and 4 studies showed response towards changes in ionic strength of the surrounding medium (14), while 1 study each described the hemicellulose-based hydrogels showed response when light radiation and magnetic-field (28) were applied to the surrounding medium. The summary of these stimuli response of hemicellulose-based hydrogels and their effects on the hydrogels performed in the studies mentioned above are shown in Table 7.

Temperature-sensitive

The hemicellulose-based hydrogels have an obvious temperature sensitivity as it was found that the SR of hemicellulose-based hydrogels was affected by changes in temperature. The SR of the hydrogels was shown to have an inverse relationship with the temperature level. Thus, increasing the temperature will resulted to decrease in SR of hydrogels. This is because when the external temperature is above the lower critical solution temperature (LCST), the network of hydrogels will shrink and became less expanded, causing the hydrogels to force out the absorbed water, and thus reducing the SR. Generally, LCST is regarded as the temperature point at which the SR of hydrogels declines sharply, and it can be manipulated by changing the ratios of polymer and/or monomer concentrations. Therefore, it permits the hemicellulose-based hydrogels to be a potential material for biomedical applications, and this include application in drug delivery system.

Ph-sensitive

Hemicellulose-based hydrogels were studied to have a pH-sensitive swelling behaviour as when the hydrogels were placed in different pH medias, they exhibit different swelling capacity. In general, the SR of hydrogels declines in an acidic pH. This is

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because the carboxyl ($-COO^-$) groups are protonated and converted into carboxylic (-COOH) groups, which led to decrease in the electrostatic repulsion forces among the $-COO^-$ groups, and increase the hydrogen bonding among the -COOH groups. This causes the network of polymer to collapse, thus reducing the SR. Meanwhile, at alkaline pH, -COOH groups are ionized and converted into $-COO^-$ groups, which increased the electrostatic repulsion forces among the $-COO^-$ groups. This resulted to the network of polymer became expanded, thus increasing the SR. The changes in SR of hydrogels at different pH values are mainly depends on the ionized pendant groups (i.e., carboxylic group), fixed charges on the network of polymer, and the electrostatic repulsive forcers. Therefore, this pH-sensitive swelling behaviour of hemicellulose-based hydrogels may make it useful material for controlled and sustained drug delivery.

Ionic strength-sensitive

Hemicellulose-based hydrogels were determined to be sensitive towards changes in ionic strength because their swelling capacity was found to be strongly depends on the ion concentrations added into the swelling medium. In a study done by Ashraf et al. it was found that SR of hydrogels decreased as the concentration of cations such as Na^+ or Ca^{2+} increased. This is because by increasing these cations concentration will resulted to decrease in the ratio of ions inside the hydrogel to the surrounding medium, and this is mainly due to the ionic interactions that took place between the cations and $-COO^-$ groups. These interactions led to the decrease in osmotic pressure between the hydrogel and water, causing the network of polymer became shrink, and forced out water from the hydrogel. Thus, reducing the SR of hydrogel. Similar findings were also demonstrated in a study by Peng et al. Moreover, when the concentration of electrolytes increased, it resulted to screening of anionic groups ($-COO^-$) of polymer network by the cations. This caused the electrostatic repulsion forces to reduce, and induced the hydrogel to shrink. Since, ions composition did influence the network structure of hydrogels, this finding may be useful in designing a more potential hydrogels preparation for drug delivery system.

Light and magneticfield-sensitive

In overall findings, temperature, pH and ionic strength are the most commonly studied stimuli for the development of stimuli-responsive hydrogels in this review. In the other hand, stimuli such as light radiation and magnetic field can also control the changes in swelling capacity of hemicellulose-based hydrogels. However, as to date, there is a very limited number of studies available on light and magnetic-sensitive hemicellulose-based hydrogels for drug delivery applications. In this review, 2 different studies had each demonstrated the response behaviour of hemicellulose-based hydrogels with the application of light radiation and magnetic-field with their significant effects on polymer matrix of the resulting hydrogels.

A study by Cao et al. on xylan-type hemicellulose hydrogels copolymerized with azobenzene was demonstrated to exhibit a light responsive behaviour. It was found that the stable trans-conformation of the azobenzene get converted into cis-conformation when the resulting hydrogels were exposed to the UV irradiation. However, this cis-conformation of azobenzene would get converted back to its trans-conformation when it was exposed to visible light or deposited in the dark. This signal changes in azobenzene conformation confirmed the trans-cis photoisomerization of azobenzene in the hydrogels, and the trans-cis photoisomerization of azobenzene resulted to the shifting of hydrophilic/ hydrophobic material of the resulting hydrogels. It was further explained that the shifting of hydrophilic/hydrophobic balance in the hydrogels is the one responsible for the drug release behaviour of the hydrogels. Therefore, light responsive hydrogels may

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have the potential for drug delivery application.

Meanwhile, a study by Zhao et al. on the development of hemicellulose-based hydrogels formed with magnetic iron oxide (Fe_3O_4) nanoparticles had shown to exhibit a magnetic responsive behaviour when exposed to the magnetic field. The study demonstrated that the Fe_3O_4 nanoparticles content formed in the hydrogels is responsible for the swelling capacity of the resulting hydrogels, in which the SR of hydrogels decreased as the Fe_3O_4 nanoparticles content increased. This is because the capability to adsorb water reduced with increasing Fe_3O_4 nanoparticles formed in the hydrogels in comparison to pure hemicellulose hydrogels, which resulted to decrease in the SR. In brief, these magnetic nanoparticles proved to be able to control the swelling capacity of the hemicellulose hydrogels, thus have potential in drug delivery application.

Drug delivery application

Aforementioned, hemicellulose-based hydrogels have an excellent tuneable swelling capacity which can be easily modified and controlled. Therefore, many recent studies were published to study the impact of this property on drug delivery applications. In this review, we have identified 15 studies that had performed drug release studies for their hemicellulose-based hydrogels to demonstrate the drug release behaviour from the resulting hydrogels prepared, and their potential applications for drug delivery system. Conclusively, different model drugs had been used in all the mentioned studies, and different potential applications were also identified in those studies. The summary of various model drugs released from the hemicellulose-based hydrogels and their applications for drug delivery system are shown in Table 8.

Controlled-release drug delivery

In this review, the drug release studies of hemicellulose-based hydrogels had shown a direct relation with the swelling ratio (SR) of hydrogels. Generally, the drug release of model drug increased as the SR of hydrogels increased. As mentioned earlier in this review, the SR of hydrogels can be controlled by changes in pH, polymer and monomer weight ratio. In other studies, application of UV radiation, magnetic field and addition of suitable pore-forming agents to the hydrogels may also influenced the SR of hydrogels. Hence, the ability of controlling the SR of hydrogels elucidate the effectiveness on controlling the drug release of model drug from the hydrogels, and serve their use in controlled-release drug delivery applications as demonstrated in 11 studies shown in Table 8, and among them had specifically showed their potential use for protein delivery (1 study), insulin delivery (1 study) and intestinal-specific drug delivery (4 studies).

Protein delivery

Protein delivery is one of many applications of hemicellulose-based hydrogels for drug delivery system. The unique properties of hydrogels making them a desirable approach in delivering protein, and this include their capability of protein adsorption and release behaviours which are useful as carrier for protein delivery. In this review, it was found that a study by Zhao et al. on the development of in situ formation of magnetic Fe_3O_4 nanoparticles during the crosslinking of hemicellulose showed that the magnetic-responsive hydrogels had a higher adsorption BSA capacity (146.5 mg/g) compare to pure hemicellulose-based hydrogels (100.2 mg/g). It was found that the Fe_3O_4 nanoparticles is the one responsible for enhancing the BSA adsorption capacity as the NH_2 groups of BSA can bind to the orbitals of Fe atom. Moreover, the BSA-loaded magnetic-responsive hemicellulose-based hydrogels had shown an effective BSA release in PBS of pH 7.2 to 7.4, with overall BSA release of 74% in 5 days. Therefore, the properties of the hydrogels with tuneable swelling capacity as well as their controllable protein adsorption and release profile had

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proven their potential application in controlled-release protein delivery

Insulin delivery

Developing novel oral insulin delivery by encapsulation of insulin in polysaccharides hydrogels in form of microspheres as an alternative for delivering insulin through oral administration has been studied by several researchers in recent years. This is because microencapsulation of insulin offers many benefits such as protection against degradation in upper gastrointestinal (GI) tract, and colon-specific drug delivery. In this review, it was shown that a study by Martínez-López et al. on the development of insulin-loaded microspheres synthesized by enzymatically crosslinking of arabinoxylan (AX) had shown that the AX microspheres were able to reduce the insulin loss in the upper GI tract during the in vitro control insulin release studies, and able to retain high percentage of insulin of approximate 75% of insulin in the hydrogel matrix. Hence, proven their effectiveness to deliver insulin via oral administration that is also colon-specific. Moreover, in vivo studies on murine to support the prior finding, were reported to have significant hypoglycaemic effects with improved insulin bioavailability, thus, promotes the effectiveness of this enzymatically crosslinked AX microspheres as an oral insulin delivery.

Intestinal-specific drug delivery

Hemicellulose-based hydrogels were proved to be a promising drug carries for the intestinal-specific oral drug delivery as they exhibited great drug release studies in response to pH changes. It was shown that drug release of model drug increased as the pH increased, thus it is ideal to be use to target the drug release specifically in the intestine. In this review, acetylsalicylic acid (ASA) had been extensively studied as a model drug for intestinal-specific drug delivery as reported in 3 out of 4 studies. This mainly due to the fact that ASA release required to be controlled differently in the gastric and intestinal fluids to overcome its major side effect, that is irritation to the stomach. Overall, in those 3 studies, the drug release behaviour of ASA from various preparations of hemicellulose-based hydrogels had shown similar findings, where the cumulative release rate of ASA was significantly higher in the intestinal fluid (pH 7.4) with 85-91% as compare to its cumulative release rate in gastric fluid (pH 1.5) with 24-26%. The mechanism of this high cumulative release rate of ASA from the hemicellulose-based hydrogels in intestinal fluid was probably due to the electrostatic expulsion forces resulting from the ionization of -COOH groups present in alkaline pH. This later caused the expansion of the hydrogel network, and led to a faster release rate of ASA from the hydrogels.

Sustained-release drug delivery

Hemicellulose-based hydrogels have appeal to be suitable drug carriers for sustained-release of encapsulated drugs in the human digestive system as reported in 4 studies. In this review, it was found that a study by Chimphango et al., on the HRP-sustained release from the enzymatically-crosslinked xylan-based hydrogels had shown that the in situ encapsulated HRP, both during and after the formation of xylan-based hydrogels was released in its active form over a period of 180 min, which indicates the resulting xylan-based hydrogels have the potential use for sustained-release drug delivery. Moreover, this study also demonstrated the release rate of HRP was influenced by in situ encapsulation methods of HRP either by after or before the formation of xylan-based hydrogels, in which the in situ encapsulated HRP before the formation of xylan-based hydrogels showed a continuous decline of HRP release rate over time as compare to the stable release rate of encapsulated HRP after the formation of xylan-based hydrogels. This condition explained that the in situ encapsulated HRP after the formation of xylan-based hydrogels had less

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restriction on the release rate of HRP from the hydrogel matrix as compare to the HRP encapsulated before the formation of xylan-based hydrogels. Thus, this finding of the stable release rate of HRP indicates that the in situ encapsulated HRP after the hydrogel formation is significant for an efficient sustained-release drug delivery, and demonstrated that the choice of encapsulation method does matter in modifying the sustained release of model drug from the resulting xylan-based hydrogels.

Conclusion

This study has successfully provided a systematic literature review of hemicellulose-based hydrogels for drug delivery system, with extensive findings on their synthesis, properties and applications for drug delivery. Conclusively, the findings of this study had shown that hemicellulose-based hydrogels have great performance and functionality, and proven to be a promising drug carrier for controlled and sustained drug release, mainly due to their great tuneable and controllable drug release behaviour which can be easily modified. Moreover, the findings of this study have covered many different hemicellulose-based hydrogel preparations available from many recent studies, which most of them presented with many great improvements to their properties which further enhance their applications for drug delivery system. Therefore, this study provides massive evidence of the development of hemicellulose-based hydrogels as a potential drug carrier for drug delivery system and may contribute to the existing scientific knowledge of biopolymers for drug delivery system, and thus promote more interest on researchers to develop a natural-based drug carrier of economical and green renewable resources, and discover a new approach on controlled and sustained drug delivery system to address the current issues or limitations with conventional drug dosage form.

Limitations

This study may have provided extensive findings, however it is still limited by the fact that there were not many studies done specifically on the drug release studies of the hemicellulose-based hydrogels with model drugs, because many studies are more focus on the development of hemicellulose-based hydrogels, and the discussion is limited to the theory of their potential use in drug delivery system. This may hinder the possibility to discover other drug delivery application that would be useful and broaden the knowledge and findings of this study. Apart from that, this study has concluded that most studies on the development of hemicellulose-based hydrogels for drug delivery system were limited to chemical crosslinking method, while there is another crosslinking method available, namely physical crosslinking method.

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Conflicts of interest

The authors declare no conflict of interest.

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Systematic Literature review on Success Factors, Issues and Challenges in Halal Assurance Management System (HAS) Implementation in the Production of Halal Products

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Abstract

Halal is becoming a global symbol for quality assurance and lifestyle choice and no longer solely for religious issue and Halal Assurance Management System (HAS) provide assurance to Muslim consumers on halal quality as both safety and quality for products and the processes are also addressed by a good quality assurance. This study was conducted by using systematic literature review guided by PRISMA protocol, where a total of 21 articles were reviewed after the searching of the keywordsfrom various databases such as Scopus, Emerald Insight, MyCite and Google Scholarand data extraction and analysis were done by thoroughly review all the included articles. Several themes were discussed according to Work System Method's element focusing on halal research trends, success factors, issues and challenges in halal industry on the implementation of Halal Assurance Management System. The findings of this study had shown that the research area covers mostly for food manufacturing industries in Small and Medium Enterprise and there are many things that need to be taken into consideration, especially in current practice and arising issues and challenges in halal industry and success factor in implementing the Halal Assurance Management System. It is advisable for manufacturers to closely follow the updated guidelines observed in the production and manufacture of halal products.

Keywords: Halal; Halal Assurance Management System; Halal Products; Production; Manufacturing

Introduction

The terms halal defines as anything that is permissible and lawful according to Shariah law. Conventionally, halal and haram concept is always applied to food and beverages but due to the effect of globalization, the scope of halal and haram encompasses not only food and drink but also othermatters of daily life including cleaning agents, cosmetics, pharmaceutical and finance(1). Halal Assurance Management System (HAS) is a system, which must be implemented by a manufacturing company to assure the halal status of the products produced(2). The halal assurance guideline should be followed by the halal certificate holders in order to meet halal certification requirements, halal regulations and standards (3). Halal products generally can be categorised into food and beverages, cosmetics, pharmaceuticals, consumer goods, medical devices and also services. The demand for halal products will continue to grow not only in Malaysia but around the world as halal products are suitable for consumption by anybody, regardless of religious beliefs(4). To manufacture halal pharmaceuticals, the Halal Assurance Management System (HAS) shall further ensure that pharmaceutical products are designed and developed in accordance with halal and Good Manufacturing Practice's requirement (GMP)(5).

Halal certification in Malaysia uses Malaysia Halal Management System Manual 2020 (MHMS 2020) as a reference to implement HAS, which contains details for implementation of two halal management systems which is Internal Halal Control

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System (IHCS) and Halal Assurance Management System (HAS). HAS is for medium and large enterprises, comprises of ten elements to adopt which are Halal Policy, Internal Halal Committee, Halal Risk Control, Halal Raw Material Control, Halal Traceability, Halal Internal Audit, Halal Training, HAS Management Review, Laboratory Analysis, and *Sertu* (*Islamic Cleansing*) program, whilst for micro and small industries are now are required to adopt Internal Halal Control System (IHCS) which comprises of three elements in its adoption which is Halal Policy, Raw Material Control/ Halal Risk Control and Traceability. HAS has become important in the halal production process because by implementing this system, the company or applicant must ensure every requirement for Malaysia Halal Certification is being complied to, thus it can maintain the sustainability of the whole process(6).

Halal certification is issued by the Government, whilst in other countries, their certification is endorsed by the respective Islamic associations. Malaysia Halal Standard, the MS 1500:2019 Halal Food – General Requirements is developed by the Government to have a clear and practical guideline for Halal compliance and to make sure only Halal food is produced(2). Halal certification bodies in charge in Malaysia comprises of federal and state level authorities which is Department of Islamic Development Malaysia (JAKIM) and State Islamic Religious Department (JAIN).Manual Procedure for Malaysia Halal Certification (MPPHM) is the official reference document for halal certification which is concurrently used together with the Malaysia Halal Standards(7).

A management system is the set of interrelated elements used by an organisation to establish the organisation's structure, roles and responsibilities, planning, operation, policies, practices, rules, beliefs, objectives and processes to achieve those objectives. Quality management system is a part of this management system from quality perspective,whereas work system is defined as systems that exist to produce products or services for their customers(8, 9). The work system method is a flexible systems analysis and design method for business professionals that uses the concept of "work system" as a focal point for understanding, visualizing, analysing, and improving systems in organization. The WSM consists of nine basic elements of a work system that comprises of processes and activities, participants, information, technologies, products and services, customers, environment, infrastructure and strategies (9). These elements are helpful to extract information needed regarding the research topic which focusses on the processes and activities in production of halal products, individuals or organization and technology involved that is implemented by the participants in the work system.

Methodology

Sampling method

The method used to retrieve articles relate to Halal Assurance Management System (HAS) from different resources that includes Scopus, Emerald Insight, My Cite and Google Scholar to run the systematic review, eligibility and exclusion criteria, steps of the review process; identification, screening, eligibility. Then it is followed by data abstraction and analysis using Atlas.ti 8.

PRISMA

The Preferred Reporting Items for Systematic reviews and Meta-Analyses (PRISMA) statement, was first published in 2009, designed to help systematic reviewers transparently report why the review was done, what the authors did, and what they found. PRISMA 2009 is a reporting guideline which aimed to address poor reporting of systematic reviews and it comprised a checklist recommended for reporting in systematic reviews (10). This standard suggested several key points to be included as stated in PRISMA checklist such as title, abstract, methods, results, discussion and

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funding sections. PRISMA offers three benefits which are: (1) clearly defines research questions; (2) identifying the inclusion and exclusion criteria, and (3) it attempts to assess and examine large amount of available and relevant scientific literature in a defined time (11).

Resources

Four main databases were used for this review, which were Scopus, Emerald Insight, MyCite and Google Scholar. The main research databases used for this study were Scopus and Emerald Insight. Scopus allows researcher to have access to search the database containing past and present articles and Emerald Insight contains high-quality journal articles, peer-reviewed research that cover a range of subjects within business, tourism, marketing and health and social care. The third database was MyCite which covers scholarly journals published in Malaysia. The other supporting database was Google Scholar, that allow researchers to find variety of materials including journal articles, books, "grey literature" like conference proceedings, non- journal that covers wide-ranging fields and it is simple to use, just like Google. However, Google Scholar may vary in quality, thus it should not be the only source used. All Scopus, Emerald Insight, MyCite and Google Scholar can use two search modes which are Basic and Advanced option however the advance search technique may vary for each database.

Eligibility and exclusion criteria

Several eligibility and exclusion criteria were determined. First, in regard of document types, only research articles with empirical data were selected. Meanwhile, other types of documents such as book chapters, conceptual articles, descriptive articles, review articles, newspapers, articles based on the analysis of the secondary sources and ethnographic and historical accounts were all excluded for this study. The next criteria for the selection criteria was language. In order to avoid any confusion and difficulties in translations work in this study, only English and Malay language documents were included. Abstracts and studies which were written in languages other than English and Malay were excluded. The third criterion is the availability of the full text articles. Due to the limited of Halal Assurance Management System (HAS) articles publication, research papers and proceeding papers were included for this review. Final inclusion criteria were the timeline, all documents published were selected without restriction of time period. The criteria are as shown in (Table 1).

Systematic literature review process

Four stages were involved in the systematic literature review process. The review process was performed in December 2021. The first phase was identifying the keywords using search string intended to be use in the selected database using several relevant information sources

Table 1: Eligibility and exclusion criteria

Criteria	Inclusion	Exclusion
Document type	Journals (research articles)	Book chapters, conceptual articles, descriptive articles, review articles, newspapers, articles based on the analysis of the secondary sources and ethnographic and historical accounts
Language	English and Malay	Non-English and Non-Malay
Accessibility	Full text article	Non-full text article
Timeline	No restriction of time period	

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such as dictionaries, thesaurus, similar keywords and previous studies that was related to Halal Assurance Management System (HAS). In the identification process, 254 documents from Scopus, 131 documents from Emerald Insight, 88 documents from MyCite and 602 documents from Google Scholar were found. Next was the screeningprocess where inclusion and exclusion criteria were decided to search for suitable articles to be included in the review process. Before the screening process was carried out, duplicate documents were first removed. The duplicated documents were removed manually by comparing all the documents in four databases and removed using colour coded system to identify the similar documents between databases and within database. A total of 67 duplicated documents were traced as duplicated articles which resulted in 1,008 documents left to be screened. Next steps in screening process are to manually select documents that meets the selection criteria. Out of 1,008 articles eligible to be reviewed, a total of 939 articles were removed because they do not meet the inclusion criteria and the rest were articles that were inaccessible due to lack of access. The third stage is eligibility, where the articles were accessed. The articles were screened manually for literature focusing on Halal Assurance Management System (HAS) and criteria from the earlier screening processes (inclusion and exclusion criteria). Any papers which do not involve the study on Halal Assurance Management System (HAS) were excluded. Out of 69 full-text articles left for eligibility process, a total of 48 were removed. The last stage of reviewresulted in a total of 21 articles included for this review that were used for the qualitative analysis. Figure 1 belowdepicts the PRISMA diagram for the data processing of the collected papers.

Data extraction and analysis

The remaining data collected for this study were assessed and analysed using qualitative thematic analysis with Atlas-ti 8 software to convert data into themes and subthemes that were adapted from Work System Method by Steven Alter. The articles were analysed to answer the research questions and the data extracted by reading through the abstracts, then full text article to identify relevant themes and subthemes related to Halal Assurance Management system (HAS).Atlas.ti is a qualitative data analysis (QDA) software, that provides some very useful tools in academic research, mainly for social science disciplines. Atlas.ti can handle not only text data but also other digital media formats like video and images (12).

The first stage of qualitative data analysis is usually open coding. Open coding is the processes of analysing textual content used to analysed qualitative data. Reviewer can choose to do Axial Coding (where the coding is organised into certain categories to address the research objectives) and Selective Coding once reviewer finish Open Coding, depending on the methods being use. Any sentences or paragraphs that can answer the research question will be selected and open coding will be added depending on the article analysis. The memo will then be linked to the coding system in accordance with the research questions. Finally, all of the linked coding and memos can be organised into a network diagram, with all of the related questions connected to one another. Work system method's elements was used to generate the main themes when analysing the data which were; 1) Customers, 2) Products or Services, 3) Process and Activities, 4) Participants, 5) Information, 6) Technologies, 7) Environment, 8) Strategies and 9) Infrastructure.

Data collection

Data collection protocol

Methods of identifying the keywords used advance search techniques which were Boolean Operator OR (/) AND (+) NOT (-), Phrase Searching"..", Truncation* and Wildcards *. Scopus supports all the advance

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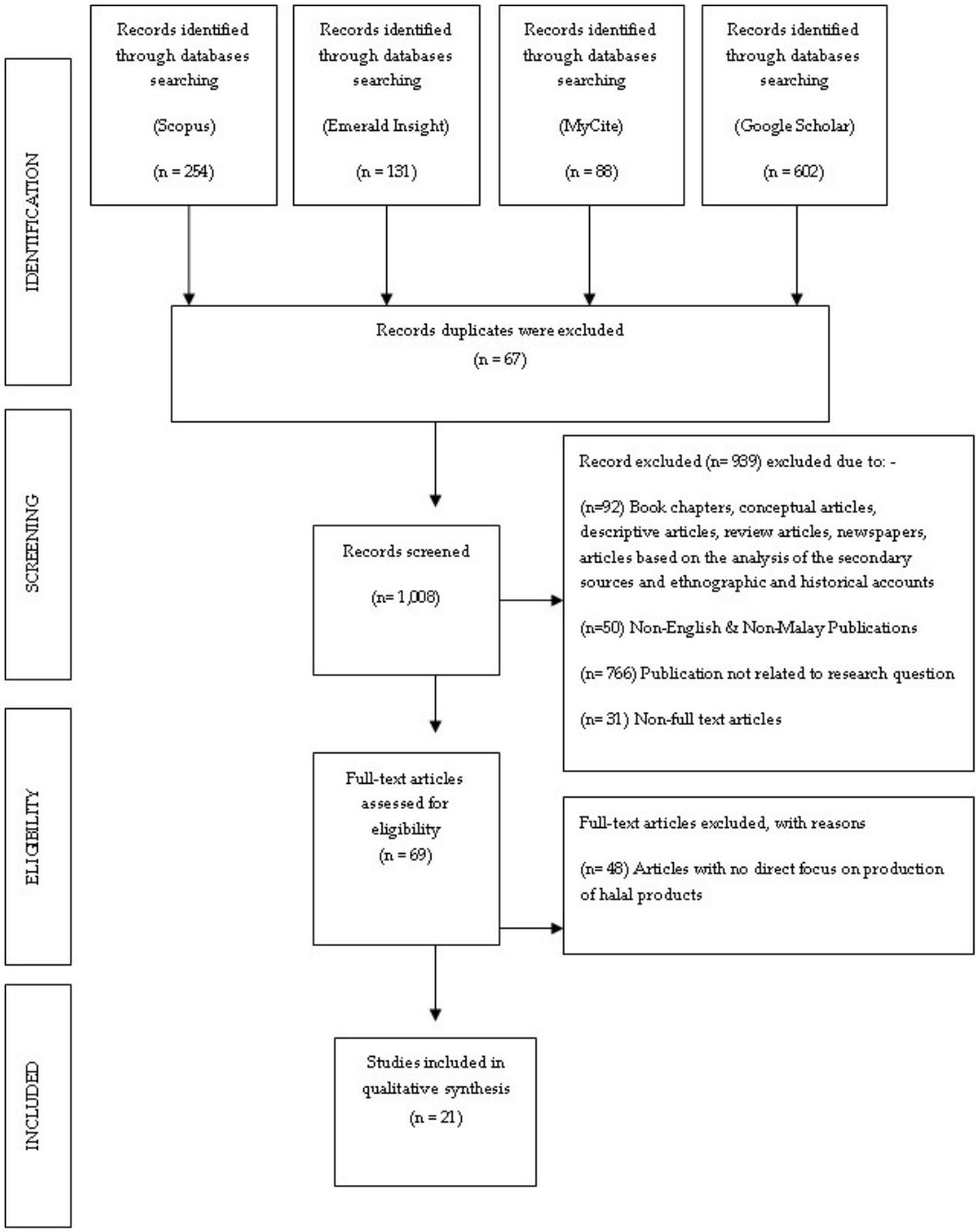

Figure 1: Flow diagram following the PRISMA guidelines

Review on Success Factors, Issues and Challenges

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Table 2: Keywords and search items for documents retrieval

Databases	Keywords and search items used for systematic searching
Scopus	TITLE-ABS-KEY (("halal" OR "s*aria* complia*" OR "s*aria*" OR "islam") AND ("assurance management system" OR "assurance system" OR "quality management" OR "built-in" OR "risk management" OR "islamic manufacturing practice") AND ("production*" OR "manufactur*" OR "mak*" OR "develop*")
Emerald Insight	(content-type:article) AND (“halal assurance management system” OR (“halal assurance system”) OR (“halal quality management”) OR (“halal management”) OR (“halal built-in”) OR (“halal risk management") OR ("Islamic manufacturing practice”) - (slaughter*) - (tourism*) - (restaurant*)
MyCite	halal assurance management system OR halal assurance system OR halal quality management OR halal management OR halal built-in OR halal risk management OR Islamic manufacturing practice
Google Scholar	• "Halal Assurance Management System" OR "halal assurance system" OR "Halal Quality Management" OR "halal management" OR "Halal Built in" OR "Halal risk Management" OR "Islamic Manufacturing Practice" -slaughter -penyembelihan -broiler -chicken -tourism • “shariah compliance production” OR “shariah compliance manufacturing” OR “shariah compliance making”

search technique include Boolean Operator, Phrase Searching, Truncation and Wildcards. Next, Google scholar uses Boolean Operator, Phrase Searching and symbol ‘-‘ (NOT). In addition, Emerald Insight support advanced search that helps reviewer to find relevant articles such as Phrase searching, Boolean search and also support Truncating whilst MyCite only support Boolean Operator and Phrase Searching. Emerald insight uses important keyword that was entered in the search box. Combination of all of these options created the advanced searching.

Keyword development

The search keywords used for the documents search are as in (Table 2). The retrieved data were screened using inclusion and exclusion criteria. Relevant literatures werecollected and extracted, subsequently analysed and tabulated into a data collection spreadsheet as in (Table 3).

Results and Discussion

Findings

A total of 1,075 publications are obtained by using databases such as Scopus, Emerald Insight, MyCite and Google Scholar. Out of 1,075 publications obtained, 21 publications were analysed. The results analysed are presented into three different sections namely, current practice in the implementation of halal assurance management system, success factor and issues and challenges. The results in each section are explained descriptively categorized into several different themes and a number of sub-themes under different sections; research trend, success factors, issues and challenges.

Trends of halal research on has

There were 21 articles obtained from the data analysis and their summaries are as presented in (Table 3, Table 4, Figure 2, Table 5, Figure 3, Table 6, Table 7 and Table 8) based on the number of papers collected by databases, years they were published, country of publisher, type of articles reviewed and scope of study in the literature. The complete information spreadsheet of the 21 articles analysed (refer table 3).

Number of paper collected by databases

Several keywords were generated to refine the systematic searching when collecting

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Table 3: List of Literature Included in Data Analysis

No	Database/ Type of Publications	Authors (Year)	Title of Article	Name Of Journal	Study Setting	Type of Study/ Research Method	Research Area	Data Collection Tool	Relevant Findings
1	Scopus /Journal Article	Nurdiyana Nazihah Zainal, Siti Fairuza Hassam, Mohd RizaimySh aharudin, Jamaludin Akbar, Muhamma d Amin Mustafa (2018)	Contributin g factors of production performan ce in the food processing industry	Internatio nal Journal of Supply Chain Manage ment	Malaysi a	quantitati ve	Food processin g manufact urers	Questionn aires survey	This article is discussing on the factors that can contribute to the production performance in the food manufacturing industry. Total Quality Management (TQM), Lean Management (LM), and Hazard Analysis and Critical Control Point (HACCP) are the factors that influenced the production performance.
2	Scopus /Journal Article	Azmawani Abd Rahman, Hassan Barau Singhry, Mohd Hizam Hanafiah, Mohani Abdul (2016)	Influence of perceived benefits and traceability system on the readiness for Halal Assurance Systemimpl ementation among food manufactur ers	*Food Control*	Malaysi a	quantitati ve	Food manufact urers	Questionn aire	This article is discussing on traceability systems that are fully needed to bridge the connection between perceived benefits and readiness for HAS.

(*Contd.)*

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Table 3: List of Literature Included in Data Analysis (*Contd.)*

No	Database/ Type of Publications	Authors (Year)	Title of Article	Name Of Journal	Study Setting	Type of Study/ Research Method	Research Area	Data Collection Tool	Relevant Findings
3	Scopus /Journal Article	Muhammad Haziq Hassan, Sazelin Arif & Safiah Sidek (2015)	Knowledge and Practice for Implementing Internal Halal Assurance System among Halal Executives	Asian Social Science 11(17)	Malaysia	qualitative study	halal food premises	interview technique	This article is investigating on the knowledge and skills of the implementing the IHAS among the executives at food premises.
4	Emerald Insight /Journal Article	Hayati Habibah Abdul Talib & Khairul Anuar Mohd Ali and Fazli Idris (2014)	Critical success factors of quality management practices among SMEs in the food processing	Journal of Small Business and Enterprise Development (JSBED)	Malaysia	questionnaire mailed	Food processing industry	survey	This article identified a few critical success factors of quality management practices proposed for assessing quality management practices among SMEs in the food processing industry in Malaysia.
5	Google Scholar /Journal Article	Mary Jane Alvero, Imee C. Acosta, Eduardo Parra Malagapo (2019)	Impact of Halal Assurance Management System on Halal Products	Middle Eastern Journal of Development Management	Philippine	quantitative	Halal products	Survey	This article analyses the impact that the halal assurance management system has on halal products based on international standards.

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Table 3: List of Literature Included in Data Analysis (*Contd.)*

No	Database/ Type of Publications	Authors (Year)	Title of Article	Name Of Journal	Study Setting	Type of Study/ Research Method	Research Area	Data Collection Tool	Relevant Findings
6	Google Scholar /Journal Article	Baharudin Othman, Sharifudin Md Shaarani, ArsiahBahr on (2016)	Evaluation of knowledge, halal quality assurance practices and commitme nt among food industries in Malaysia	British Food Journal	Malaysi a	quantitativ e	food industries	questionnai re	This article analyses level of knowledge, halal quality assurance practices and commitment among food industries in the implementation of halal in Malaysia.
7	Google Scholar /Journal Article	Baharudin Othman, Sharifudin Md. Shaarani, ArsiahBahr on, Nurul Hudani Md Nawi (2019)	The Influence of Halal Practices on Organizatio nal Performanc e Among Food Industries (Smes) In Malaysia	Halal Journal	Malaysi a	quantitativ e	halal food industry (SMEs)	closed-ended questions questionnai res cross-sectional surveys method.	This article analyses how the key elements as performance predictors influence the performance of the organization as compared to internal dimensions of the process and staff.
8	MyCite /Journal Article	Mohd Zabiedy Mohd Sulaiman, Nurulhuda Noordin, Nor Laila Md Noor, Ahmad Iqbal Hakim Suhaimi, Wan Abdul Rahim Wan Mohd Isa (2019)	Halal Virtual Inspection Critical Control Point	Internatio nal Journal on Perceptiv e and Cognitive Computin g (IJPCC)	Malaysi a	Qualitativ e	small and medium-sized food premises	Interview &Observaio n	This article discussing on the critical control point (CCP) of Halal Inspection (HI) by focusing on the inspection process and several requirements for the HI process for small and medium-sized food premises conducted in Malaysia.

(*Contd.)*

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Table 3: List of Literature Included in Data Analysis (*Contd.)*

No	Database/ Type of Publications	Authors (Year)	Title of Article	Name Of Journal	Study Setting	Type of Study/ Research Method	Research Area	Data Collection Tool	Relevant Findings
9	Google Scholar /Journal Article	Zurina Shafii, Siti Norfaizzah Zubir, Norafni @ Farlina Rahim (2018)	Halal Governance and Assurance : A Comparative Study Between Malaysia And Thailand	International Journal of Islamic Economics and Finance Research	Malaysia and Thailand	Qualitative	Halal industry	interviews, audit documents and observation of audit process	This article is studying the comparison between Malaysia and Thailand on their Halal governance and also on this aspect which is Halal regulator, regulations, auditors, validity of certification, scope of audit, frequency of audit, and the flexiblity of Halal logo usage.
10	MyCite /Journal article	Sumaiyah Abd Aziz, Mohd Mahyeddin Mohd Salleh, Mustafa 'Afifi Abdul Halim &Hasdhatul Nor Aliah Md Said (2021)	Amalan TerbaikPelaksanaan SertudalamIndustri Halal di Malaysia (Best Practices of Islamic Cleansing (Sertu) Implementation in the Malaysian Halal Industry)	Journal of Fatwa Management and Research (*JFATWA*) (Jurnalpengurusan dan penyelidikan fatwa)	Malaysia	qualitative	Islamic cleansing (sertu) in the Malaysian halal industry - medium and large industries	face-to-face interviews and direct observation.	The aim of this study is conducted to look at the best practices for implemention of Islamic cleansing (sertu) in the Malaysian's medium and large industries halal industry as sertu is one of the halal requirements in the HAS, Malaysian Halal Management System (MHMS) 2020.

(*Contd.)*

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Table 3: List of Literature Included in Data Analysis (*Contd.)*

No	Database/ Type of Publications	Authors (Year)	Title of Article	Name Of Journal	Study Setting	Type of Study/ Research Method	Research Area	Data Collection Tool	Relevant Findings
11	Google Scholar /Journal Article	Maresta Andriani, Ida Giyanti, dan Anita Indrasari (2020)	Proposed Improvement of Standard Operating Procedure According to Halal Standards at Siska Bakery	Performa : Media Ilmiah Teknik Industri (2020)	Indonesia	qualitative	Food products	Interview, observation and document review.	This research intends to create SOPs that include the purchasing of raw materials, production, and product distribution in order to meet the halal standards outlined in the HAS 23000 document.
12	Scopus /Journal article	Kim Hua Tan, Mohd Helmi Ali, Zafir Mohd Makhbul, Azman Ismail (2017)	The impact of external integration on halal food integrity	Supply Chain Management: An International Journal	Malaysia	quantitative	Malaysian halal food manufacturing firms	survey	This article is discussing on the impact of external integration on compliance with halal standards, for the integrity of food products within the food industry and the links between external integration and halal assurance system.
13	Google Scholar /Journal Article	Sucipto Sucipto, Alda Alvita, Luki Hidayati, Muhammad Arif Kamal,Retno Astuti, Nur Hasanah (2021)	Assessment of Knowledge, Skills, and Attitudes of Trainees of Halal Assurance System Training in Micro and Small Food Enterprises	Jurnal Agroindustri Halal	Indonesia	qualitative method and quantitative	Micro and Small Food Enterprises	questionnaires	The study is conducted to evaluate the HAS implementation training programme for MSEs focusing on the trainees' satisfaction, knowledge, abilities, and attitude.

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Table 3: List of Literature Included in Data Analysis (*Contd*.)

No	Database/ Type of Publications	Authors (Year)	Title of Article	Name Of Journal	Study Setting	Type of Study/ Research Method	Research Area	Data Collection Tool	Relevant Findings
14	Scopus /Conference paper	Anita Indrasari, Ida Giyanti,Wahyudi Sutopo, and Eko Liquiddanu (2019)	Halal assurance system implementation and performance of food manufacturing SMEs: A causal approach	The 5th International Conference on Industrial, Mechanical, Electrical, and Chemical Engineering 2019	Indonesia	qualitative method	Food Manufacturing SMEs	Observation	This paper discussing on the link between halal assurance system (HAS) implementation and performance of food manufacturing SMEs.
15	Scopus /Conference paper	C G Perdani, N U Chasanah and Sucipto (2018)	Evaluation of halal assurance system (HAS) implementation on bakery products processing in small and medium enterprises (case study in X Bakery Batu, East Java)	International Conference on Green Agro-industry and Bioeconomy	Bakery Batu, East Java, Indonesia	quantitative	bakery products processing in small and medium enterprises	Interview method	This article evaluates HAS applied by SMEs in bakery product processing accordance to Indonesian Halal Assurance System.
16	Scopus /Conference paper	Achmad Samudra Dewantara, Eko Liquiddanu, Cucuk Nur Rosyidic (2018)	Assessment of the readiness of SME to entering the modern market by using the good manufacturing practice and halal assurance system (Case study on Sari Murni SME)	The 3rd International Conference on Industrial, Mechanical, Electrical, and Chemical Engineering	Indonesia	quantitative	Tofu - Sari Murni SME	1. observation at The Sari Murni - questionnaire	This article is discussing a case study of assessment of the performance of halal assurance system and level of the non-conformity of good manufacturing practice (GMP) in Sari Murni (SM) which is the SME that produces tofu in Indonesia.

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Table 3: List of Literature Included in Data Analysis (*Contd.)*

No	Database/ Type of Publications	Authors (Year)	Title of Article	Name Of Journal	Study Setting	Type of Study/ Research Method	Research Area	Data Collection Tool	Relevant Findings
17	Scopus /Conference paper	Mohd Zabiedy Mohd Sulaiman, Nurulhuda Noordin, Nor Laila Md Noor, Ahmad Iqbal Hakim Suhaimi, Wan Abdul Rahim Wan Mohd Isa (2018)	Halal virtual inspection requirements for food premise inspection process - Towards the virtualization of Malaysia Halal certification system	International Conference on Information and Communication Technology for the Muslim World	Malaysia	Qualitative method	Food Premise	1. Interview - open-ended interview questions, interview was voice recorded 2. Observations	This article is discussing on the critically important requirement of Halal Inspection (HI) which is the key process of Halal Certification (HC) system.
18	Google Scholar /Conference paper	Mirajiani, Wahyu Susihono (2021)	The Evaluation of Has-23000 Implementation in Sate Bandeng Industry Certified of Halal	Joint proceedings of the 2nd and the 3rd International Conference on Food Security Innovation (ICFSI 2018-2019)	Banten Province, Indonesia	Qualitatve method	Sate Bandeng industry from Banten Province	1. Observations 2. Interviews	This article aims to determine the level of application of HAS-23000 which is a standard from LPPOM MUI in Indonesia, in halal certified milkfish satay industry in Banten Province, and the result was used for basic policy making.
19	Google Scholar /Conference paper	Baharudin Othman, Sharifudin Md. Shaarani, ArsiahBahron (2016)	The Effect of Halal Requirement Practices on Organization Performance Among Food Manufactures in Malaysia	23rd International Academic Conference, Venice	Malaysia	quantitative research	Food manufacturers	cross-sectional surveys method. questionnaire	This article focused on the halal requirement practices that have impact on organizational performance in Malaysian halal food industry.

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Table 3: List of Literature Included in Data Analysis (*Contd.)*

No	Database/ Type of Publications	Authors (Year)	Title of Article	Name Of Journal	Study Setting	Type of Study/ Research Method	Research Area	Data Collection Tool	Relevant Findings
20	Google Scholar /Conference paper	Della Ika Aldista, David Atmaja, Johanes Kurniawan, Riza Lestari (2018)	The Effects of The Implementation Of Halal Critical Activities On The Quantity Of The Order Received By Company Which Has Run Iso - Case Study: Pt Ych Indonesia	Advances in Engineering Research (AER), volume 147 - Conference on Global Research on Sustainable Transport (GROST 2017)	Indonesia	qualitative	COMPANY WHICH HAS RUN ISO	1. interviews with employees or telephone interviews with the secretary 2. documents related to halal certification 3. questionnaire	This article is discussing on the similarities between halal and ISO 9001-2008 in case study PT YCH Indonesia that guarantee halal status in handling until reaching the customers.
21	Scopus /Conference paper	Nurulhuda Nordin, Nor Laila Md Noor, and Zainal Samicho (2012)	Applying the Work Systems Method to Investigate the Operational Efficiency of the Halal Certification System	19th IBIMA Conference	Malaysia	qualitative	halal suppliers and manufacturers	face to face interviews	This study aims to identify halal certification processes by using the work systems method as a framework for inquiry in order to provide a holistic view of the halal certification environment using the Malaysian halal certification system as the case study.

the studies with regard to the research question. Each keyword is specifically used in each of the databases. Table 4 shows the number of papers collected from each database.

Number of publications by year

The publication year of articles reviewed were not limited by the year they were published. There were no paper prior to 2012, and there are three papers published

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Table 4: Number of papers by selection of keywords

Database	Number of papers (n= 1,008)	Number of papers that can be used (n= 21)	Articles no. in data collection spreadsheet
Scopus	251	9	1, 2, 3, 12, 14, 15, 16, 17 and 21
Emerald Insight	130	1	4
MyCite	84	2	8 and 10
Google Scholar	543	9	5, 6, 7, 9, 11, 13, 18, 19 and 20

Table 5: Number of Publications According to Year

Year	Number of papers (n=21)	Publications no. in data collection spreadsheet (Table 3)
2012	1	13
2013	0	-
2014	1	14
2015	1	15
2016	3	16-18
2017	1	19
2018	6	20-25
2019	3	26-28
2020	2	29,30
2021	3	5, 31 and 32

Table 6: Number of Paper According to the Country of Publisher

Country of publisher	Number of paper (n=21)	Publications no. in data collection spreadsheet (Table 3)
Malaysia	12	5, 13-19, 25-28
Indonesia	7	20-22, 29-32
Malaysia and Thailand	1	23
Philippine	1	26

in 2021 that is related to the topic of this study. As shown in Table 5, the year 2018 have the most published papers regarding Halal Assurance Management System in Production of Halal Products.

Regarding the year of publication, one article was published in 2012, one article was published in 2014, one paper was published in 2015, three studies were published in 2016, one article was published in 2017, six papers were published in 2018, three papers were published in 2019, two articles were published in 2020and three papers were published in 2021.

Number of paper published according to the country of publisher

Four countries such as Malaysia, Indonesia, Thailand and Philippine are involved in publishing these 21 publications.

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Table 7: Classifications of the Type of Publication Reviewed		
Types of publications	Number of publications (n=21)	Publications no. in data collection spreadsheet
Journal article	12	1, 2, 3, 5, 6, 7, 8, 9, 10, 11, 12, 13
Conference paper	8	14, 15, 16, 17, 18, 19, 20 21
Research paper	1	4

Table 8: Research Methodologies and Type of Study used in The Literature			
Research Methodology	Type of Study	Number of publications (n=21)	Articles no. in data collection sheet (Table 3)
Survey	Quantitative	9	1, 2, 4, 5, 6, 7, 12, 13, 19
Interview+ Observation	Qualitative	5	8, 10, 11, 17, 18
Interview	Qualitative	3	3, 15, 21
Observation	Qualitative	2	16, 14
Interview + Audit documents +Observation	Qualitative	1	9
Interview + Document review + Survey	Qualitative+ Quantitative	1	20

From the results, Malaysia is the leading country of publisher with 12 papers followed by Indonesia with 7 papers, Philippine with 1 paper and lastly Malaysia together with Thailand with 1 paper. Table 6 below shows the country of publisher involved in publishing the articles reviewed.

Classification of the type of publication reviewed

The articles are classified into threetypes; journal article, research paper, case study and conference paper. Type of publication with the highest number of papers is journal articles with 12 papers. Table 7 shows the classification of the type of articles reviewed.It was recorded that 12 publications were journal articles, one research paper, and eightconference papers.

Research methodologies used in the literature

Four research methodologies such as survey, interview, observation, audit documents and document review were used in the publication reviewed. Table 8 below shows the research methodologies and type of study which is quantitative and qualitative study used in the publication reviewed. Survey is the main choice for research methodology with nine publications and qualitative study is the major type of study with eleven publications.

Success factor of halal assurance management system implementation

Success factor is vital in order to make sure that Halal Assurance Management System (HAS) can be implemented successfully in halal industry. Eight themes will be discussed under this section which are customer, product and services, process and activities, participants, information, technologies, environment and strategies. First factor was customers's trust. In order to gain customer's trust in their halal products, it is important to implement halal assurance system as it has a significant

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impact of halal products. Nowadays, customers are concerned about the quality safety of the product that they bought. The quality and safety of a product's requirement could be met by implementing Good Manufacturing Practice (GMP) into the production process and also by applying halal certification to the products. In the process theme, according to study by Rahman et al.(18), SMEs manufacturers must create and implementing traceability system to have a sustainable Halal Assurance System and to help the halal product's tracking process in order to adhere to HAS principle and practices. Traceability system is important as it is able to identify and trace raw materials and products through each stage include receiving, processing, storage, distribution and others along the halal supply chain. Well-organised administration and documentation system would facilitate the tracing process if there is any problem during halal production. Traceability system implementation would be one of the success factors as it can attract new consumer and meet expected future customer requirements, meanwhile it can benefit the company implementing it as it can decrease costs and increase their performance too. Furthermore, food preparation specified based on halal certificate and IHAS can produce high quality food as it can guarantee the use of halal raw material certified by the authorities. Next, it is not only necessary to commit to cleaning process and have harmonized cleaning procedure, it is also important to emphasize the employee's health in the manufacturing process. Apart from that, training and education on the implementation of halal assurance system is important to be conducted among the personnel as it can improve their knowledge and skills to comply to the procedure when they are working. In order to make sure the product is stored and shipped according to its quality; halal logistics too play an important success factor to maintain the product quality.

There has been a study by Perdani et al., (22), that shows HAS can be implemented when there is a good halal team as the top management that should be institutionalized and following halal policy. In addition, having trained Halal Auditors and halal executive that have comprehensive understanding on the system can also contribute to a successful HAS implementation. It is proven that knowledge and understanding of the personnel when adopting the management system can contribute to quality, safety and hygienic production as one of the major success factors. Moreover, halal product producers and manufacture must fully observe the HAS based on the standards and when the guideline is adjusted according to the current need in the market, there would always be a room for improvement that can be done by the authorities. Furthermore, operational performance can be improved when the manager in a company can integrate the HAS within the company's quality management system. Other than implementing the system, halal certification and verification is becoming a vital approach for further assurance to a particular halal brand as the halal sector has made halal certification as a part of halal assurance system since halal certification demand are growing as mentioned by Nordin et al.,(13).

Hassan et al.(15) mentioned that those food premises that implement IHAS can be more at advantage compared to premises without halal logo. IHAS is mainly use to supervise the use of raw material that is halal and certified by halal authorities and this implementation can produce high-quality raw materials and ingredient in the food preparation following the requirement of halal certificate. Moreover, a compilation of good food handling procedures based on the IHAS and implementing the quality assurance, leadership, information management, customer focus, human resource management, process management, supplier focus, and corporate planning when implementing quality management method

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also play a major role as the success factor, supported by Hassan et al. (15) and Talib et al.(14).

Besides, virtual technology is one of the modern technological advancements used for halal inspection process. Virtual technology collaboration with halal inspection team is believed to improve the existing halal inspection process and improve system's productivity and it helps the halal certification practitioners identify the critical control point (CCP) virtually by removing the difficulties during the evaluating process as mentioned by Mohd Sulaiman et al. (27). Then, Abd Aziz et al.(5) said that there is also a need to build a database for Islamic cleaning process. Finally, to satisfy the customer, it is recommended for halal business owner to improve their brand perceived quality, satisfaction, trust, and loyalty.

Issues and challenges of halal assurance management system

This part focuses on the issues and challenges that commonly arise in the halal industries when implementing Halal Assurance System in production of halal products. Four themes will be discussed which are i) process and activities, ii) participants, iii) information and iv) environment. There are numerous of issues that has been arises in halal industry, firstly in processes and activities themes, according to Othman et al.(17) for European businessman, there are few of them that are unaware of some aspect of the halal certification criteria that shows lack of awareness in the system within the businessmen. Study done by Mirajiani and Susihono, (31) mentioned that there was a lack of reporting and documented procedures in Small and Medium Industries and the procedures are also not implemented in the industries. Halal training is a specific training relating to halal designed and usually implemented by the company internally inclusive of halal awareness and halal competency. However, there are issues when there is no internal training in implementing the SJH in Indonesia and there were SJH manual that is not been created by the company because of few reasons either the system is not ready or no one in the company understands the SJH manual's requirement, thus they do not meet all of the HAS 23000 criteria supported by Sucipto et al.(32) and Perdani et al.(22). In addition, regarding Islamic cleansing (Sertu) process, there are study found that the current guideline for Sertu process is too general and they are not specific according to industries. The next one, there is an issue stated by Hassan et al.(15) that implementation of the assurance system is only implemented by the food premises' halal executives, and it is implemented in a non-systematic manner and did not follow to the IHAS' requirements. Moreover, no specific committee was appointed to take care of IHAS and supervised whether they comply with its requirements and only depended on the halal executive solely. This limited amount of manpower appointed to enforce the IHAS could affect the halal production process in the long run.

Apart from that, under the information theme, there was lack of knowledge and understanding within the halal committee for instance within Halal executives. It is found that according to Hassan et al. (15), the committee only depends on their knowledge and understanding of Islam and their previous experience in managing food premises without understanding the requirements of IHAS itself when implementing the system. When there is not enough knowledge in this area, it will lead to incomplete certification process according to paper found in Indonesia. Halal policy is a commitment statement that stated the company is determined to only produce and market halal products consistently in order to meet consumer needs and prioritize customer satisfaction. Mirajiani and Susihono, (31) mentioned that in one of the case studies conducted, a food manufacturing company does not have halal policy, and has not become the company's commitment.

Conclusion

This study has successfully provided a systematic literature review on Halal Assurance Management System in

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production of halal products. Conclusively, the findings of this study had shown that from the 21 articles there are still issues and challenges arise when implementing this system in halal industry. The majority of issues and challenges were caused by there is lacking understanding and knowledge and lack of effective halal committee within the company itself to implement the assurance system. It is proven that the effective halal committee within the top management in a company and implementation of standardized assurance management system, are needed as the backbone to the successful implementation of Halal Assurance Management System. This study may have provided some review on HAS research findings, however there are still limited information by the fact that there were not many studies done specifically on the halal assurance management and also the inaccessibility of the documents is also limited and the discussion are majority based on studies in food industries especially in SMEs industries thus limiting the studies to cover wide areas in halal industries.

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Antioxidant And Anti-Inflammatory Properties of *Annona Squamosa L.*: A Review

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Abstract

Annona Squamosa L. (Sugar Apple) belongs to the family of Annonaceae and it is a tropical and native species of Bahamas, Bermuda, Brazil, Central America, Ecuador, Egypt, India, Mexico, Peru, South America, and West India. Different parts of *Annona Squamosa* L. have been studied throughout the years for its benefits in health, medicinal and traditional uses related to the composition of various chemical compounds present in the sugar apple. The antioxidant and anti-inflammatory properties of *Annona Squamosa* L. have been researched in various research and studies for its effectiveness in treating ailments and illnesses through *in vitro* and *in vivo* assessment.

Keywords: *Annona Squamosa* L, Sugar Apple, Antioxidant, Anti-Inflammatory

Introduction

Sugar Apple (Annona Squamosa L.) Tree

Annona Squamosa L. is a tropical and native species of Bahamas, Bermuda, Brazil, Central America, Ecuador, Egypt, India, Mexico, Peru, South America, and West India [1, 2, 3, 4]. The Indian Council of Agricultural Research (ICAR) in India has reported that *Annona Squamosa L.* is widely cultivated with an overall surface area of 40,000 hectares in various countries such as Andhra Pradesh, Assam, Bihar, Chhattisgarh, Gujarat, Madhya Pradesh, Maharashtra, Rajasthan, Tamil Nadu, and Uttar Pradesh [5, 4].

Annona Squamosa L. is an edible fruit. The tree of *Annona Squamosa L.* grows from tiny sprouts as it springs 3 m up to 8 m. It has a large brownish to light brown bark with randomly spread branches and thin leaves [2, 4]. *Annona Squamosa L.* has been used as a natural remedy and in numerous food industries. For example, the pulp of *Annona Squamosa L.* is a flavouring agent in soft serve, and it's edible to be used as juice. *Annona Squamosa L.* pulp contains 35 to 42 mg per 100 g of vitamin C (Figure 1a). The dietary fibre, vitamin B1 which is thiamine, and potassium is high in *Annona Squamosa L.* [6, 4].

Sugar apple belongs to *Annona Squamosa L.* species to the Annonaceae family (Table 1) compromising, approximately 135 genera and 2300 species. *Annona Squamosa L.* might be the most tolerant towards droughts of water among the other species of the Annonaceae family as it will poorly grow and produces where the rains are frequent. It can grow with more than 700 mm of rainfall per year as temperature is the limiting factor when the frost will kill the young trees while the older trees have slight tolerance to the cold temperature. The plant sprout has high photosensitivity at 30 ^{o}C and vigorously shooting growth. The optimal soil pH for *Annona Squamosa L.* is between 6.0 - 6.5 pH. It can also grow in a variety type of soils from sandy soil to clay loams [7].

The different components of *Annona Squamosa L.* have chemical compositions that been listed down by [7]. The chemical

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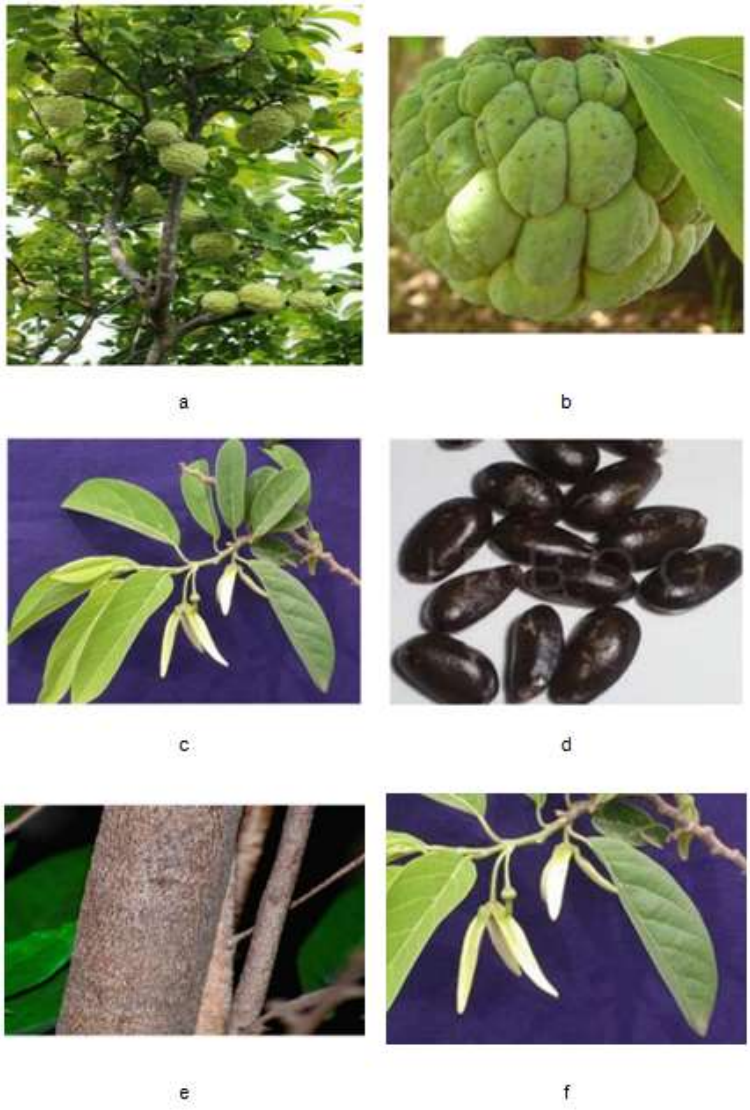

Figure 1. (a) Sugar Apple (*Annona Squamosa L.*) Tree [42]; (b) Fruit; (c) Leaves; (d) Seeds; (e) Bark; (f) Flower [7]

constituents existing in the fruits of *Annona Squamosa L.* (Figure 1b) are noorcorydine, isocorydine, liriodenine, and norushinsunine. *Annona Squamosa L.* leaves (Figure 1c) are rich in alkaloid compounds such as aporphine, roemerine, rhamnoside, norisocoryline, and quercetin-3–o-glucoside.

The bark of *Annona Squamosa L.* (Figure 1e) contains acetogenins. For example, squamone, squammotacin, annosquamosins A, B cyclopeptides, and 2, 4 cis and trans squamoxinone. The chemical compounds that available in the seeds of *Annona Squamosa L.* (Figure 1d) are annonastatin, asimicin, and squamocin.

Properties of *Annona Squamosa L.*

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Table 1: Taxonomy of Sugar Apple (*Annona Squamosa* L.) Plant		
Taxonomical Classification:		References
Kingdom	Plantae	[2, 42]
Division	Magnoliophyta	
Class	Magnoliopsida	
Subclass	Magnoliidae	
Order	Magnoliales	
Family	Annonaceae	
Subfamily	Maloideae	
Tribe	Abrae	
Genus	*Annona* L.	
Species	*Annona Squamosa* L.	
Synonyms:		
English	Sugar apple	[43, 7]
	Custard apple	
	Sweet sop	
	Sweet apres	
	Sitaphal	
Habit and Habitat (Figure 1a): A semi-evergreen tree with a height of 3 - 8 m		[44, 7]
Morphology:		
Fruits (Figure 1b)	A round to heart-shaped, and have an ovate or conical appearance, size of 5 - 10 cm in diameter, and the pericarp have a protuberant on the surface	
Leaves (Figure 1c)	An oblong lanceolated shape, size of 6 to 17 cm long and 3 to 5 cm wide, arranged alternately on short petioles	
Seeds (Figure 1d)	Oblong, smooth, shiny, blackish to brownish colour, and 1.3 - 1.6 cm long	
Bark (Figure 1e)	Thin, greyish colour	
Flower (Figure 1f)	Fleshy, greenish, droopy, extra-axillary, leafy shoot on older bark and stem, blooms as elongated shoot	

Sugar Apple *(Annona Squamosa L.)* Medicinal Properties

Annona Squamosa L. is a multipurpose plant that has several medicinal properties such as anti-inflammatory, antioxidant, antimicrobial, cytotoxic, antiulcer, hepatoprotective, antidiabetic, antilipidemic, antitumor, vasorelaxant, anthelmintic, genotoxic, and analgesic activity [8, 9]. The medicinal properties involve the presence of bioactive constituents in the different components of the *Annona Squamosa L.* plant as it is used to treat ailments and human diseases. *Annona Squamosa L.* is also used traditionally in treating epilepsy, constipation, haemorrhage, dysentery, fever,

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ulcer, worm infection, and cardiac complications [9].

The fruits of *Annona Squamosa L.* are used as stimulants, astringent, and sedatives, have hematinic, and expectorant activities also useful in the treatment of anaemia and burns as it acts as a coolant. The seeds have anti-inflammatory activity, are used in hypotensive conditions, and haemolysis of the red blood cells, the defatted seeds' extract has antitumor and central analgesic activity. The leaves of *Annona Squamosa L.* can be used in treating hysteria and fainting spells in the form of crushed leaves, swelling, and antidiabetic activity. Its roots also have antidiabetic activity, and purgative effect, used in treating spinal bone marrow disorders, and dysentery. *Annona Squamosa* L.'s bark can prevent diarrhoea and has anticancer activity [7].

Phytochemistry of Sugar Apple *(Annona Squamosa L.)*

The pulp of the *Annona Squamosa L.* is a sweet fruit with an aromatic flavour. It contains almost 28% of sugar which includes 2.53% of sucrose as the pre-dominant sugar in the pulp, and 5.05% and 0.04% as the percentage of sugar content for dextrose and laevulose, respectively. It also contains significant amounts of amino acid, ascorbic acid, calcium, carotene, dietary fibres, iron, magnesium, niacin, potassium, riboflavin, thiamine, and vitamin C. Even with the significant amounts of sugar content in its pulp, *Annona Squamosa L.* has a low glycaemic index and a moderate glycaemic load. Specific chemical constituents have been extracted such as aliphatic ketones like palmitone, and organic acids for example hexanoic, purines, and octanoic acid [10].

An analysis of *Annona Squamosa L.* leaf and its oil by gas chromatography-mass spectrometry (GC-MS) was able to discover a total of 59 chemical compounds. The main chemical compounds present in the leaf and oil of *Annona Squamosa L.* are β-caryophyllene, a natural bicyclic sesquiterpene with a percentage of 31.4%, δ-cadinene with 6.7%, α-muurolene consists of 5.5%, α-cadinol has 4.3%, and isoquinoline alkaloids. Annoreticuin and isoannoreticuin were two acetogenins isolated and identified from *Annona Squamosa L.* leaves as they possess cytotoxic selectivity towards certain human tumours [11, 10].

Nuclear magnetic resonance (NMR) spectroscopy is used in the identification of chemical constituents in *Annona Squamosa L.* root and bark. Oxoaporphine compounds such as liriodenine and oxoanalobine are found in the bark. Different chemical constituents are found in the extract of *Annona Squamosa L.* root, for example, camphene, camphor, car-3-ene, carvone, borneol, β-caryphyllene, eugenol, farnesol, geraniol, 16-hexatriacontane, hexacontanol, higenamine, isocorydine, and limonine[12, 10]. *Annona Squamosa L.* seeds have been analysed and it has isolated thirty acetogenins such as squamocins B to N, coumarinoligans, annotemoyin-1, annotemoyin-2, glucopyranoside, squamocin, and cholesteryl as it appeared to display antimicrobial and cytotoxic activities [13, 10].

Antioxidant Properties of *Annona Squamosa L.*

Bark

[14] investigated the total antioxidant capacity of *Annona Squamosa L*. bark using FRAP assay. The bark of *Annona Squamosa L*. was extracted by air-dried method and ground into a powdered sample. Then, 0.1 g of powdered sample of *Annona Squamosa L*. bark was mixed and vortexed in 5 mL of 80% methanol for 15 minutes. The sample was heated in a water bath at the temperature of 60 °C for 40 minutes and centrifuged at 4,000 rpm for 5 minutes. The supernatant was collected in a 15 mL of centrifuge tube and the extraction procedure was repeated for the remaining procedure. The total antioxidant capacity of *Annona Squamosa L*. bark was 33.09 ± 0.54 mg TE/g DW.

[15] assessed the antioxidant activity of *Annona Squamosa L*. bark via the DPPH

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method in two different solvents which are 80% methanol and 80% ethanol. The bark of *Annona Squamosa L*. was extracted using a maceration process in 24 hours and concentrated using a rotary evaporator at the temperature of 40 °C. The IC_{50} value of the *Annona Squamosa L*. bark for ethanol and methanol extracts was 55.77 µg/mL and 38.49 µg/mL, respectively.

[16] conducted the antioxidant activity of *Annona Squamosa L*. bark in hexane solvent using the Soxhlet extraction method at 40 °C and concentrated in a rotary evaporator at the temperature of 40 °C. The extraction procedure was repeated using a methanol solvent by extracting the sample at 70 °C. The hexane and methanolic extracts of *Annona Squamosa L*. bark undergo antioxidant screening using the DPPH method. The IC_{50} value of the *Annona Squamosa L*. bark for the methanolic and hexane extracts was 70.55 µg/mL and 132.23 µg/mL, correspondingly.

Leaves

[17] evaluated the antioxidant activity of *Annona Squamosa L*. leaves. The leaves of *Annona Squamosa L*. were extracted with three different solvents; methanol, acetone and water using a maceration process with a ratio of 1:10 w/v for 48 hours. The antioxidant activity of *Annona Squamosa L*. leaves extracts were determined by the DPPH, H_2O_2, NO scavenging activity, and reducing power assay. In the DPPH assay, the IC_{50} value of the methanol, acetone, and aqueous extracts of *Annona Squamosa L*. leaves was 51 ± 1.6 µg/mL, 33.9 ± 4.8 µg/mL, and 98.3 ± 0.4 µg/mL, respectively. For the H_2O_2 and NO assay, the IC_{50} value for methanol, acetone, and aqueous extracts of *Annona Squamosa L*. was 735 ± 49.5 µg/mL, 516.7 ± 5.8 µg/mL, 110 ± 14.1 µg/mL, 12 ± 4.2 µg/mL, 44 ± 5.7 µg/mL, and 81 ± 1.4 µg/mL, accordingly. The reducing power assay shows that the aqueous extract (0.984 µg/mL) has the highest reducing power followed by the methanol extract (0.975 µg/mL) and the acetone extract (0.950 µg/mL) at the concentration of 0.75 mg/mL of *Annona Squamosa L*. leaves extracts.

[18] analysed the antioxidant activity of different solvent extracts from *Annona Squamosa L*. leaves through the DPPH method. There are six different solvent extracts used in screening the antioxidant activity of *Annona Squamosa L.* leaves which are acetone, chloroform, hexane, methanol, petroleum ether, and aqueous solvent. The powdered form of *Annona Squamosa L*. leaves were extracted by maceration extraction method in the six different solvents with a ratio of 1:10. The extracts were filtered and vaporised at 40 °C to form solid extracts. The IC_{50} value of *Annona Squamosa L*. leaves for methanol, aqueous, chloroform, petroleum ether, acetone, and hexane were 96.09 ± 1.3 µg/mL, 148.09 ± 1.2 µg/mL, 234.69 ± 0.5 µg/mL, 361.22 ± 0.7 µg/mL, 396.43 ± 0.9 µg/mL, and 438.79 ± 0.1 µg/mL, respectively.

[14] determined the capacity of total antioxidants for the leaves of *Annona Squamosa L*. via the FRAP assay. *Annona Squamosa L*. leaves were air-dried, ground into powder, and extracted in 5 mL of 80% methanol. The sample was heated at 60 °C in a water bath for 40 minutes and centrifuged at 4,000 rpm for 5 minutes. The supernatant was separated into a centrifuge tube and the sediment undergoes an extraction process. *Annona Squamosa L*. leaves have a total antioxidant capacity of 53.39 ± 0.48 mg TE/g DW.

[19] explored the antioxidant activity of essential oil from *Annona Squamosa L*. leaves via the DPPH and FRAP assay. The leaves were shade dried, ground into powder and divided into two groups. The first group of leaves was stored at an ambient temperature in a dark environment within one year and the second group undergo an extraction process to obtain the first sample of essential oil. Subsequently, the first sample of essential oil was stored in a chiller at -18 °C for one year obtaining the second sample of essential oil. The third sample of essential oil was obtained directly from the

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extraction process of the first group of leaves. The powdered form of *Annona Squamosa L*. leaves undergo hydro-distillation extraction method using the Clevenger apparatus for 3 hours. The antioxidant activity via the DPPH method for the first, second and third samples of essential oil from *Annona Squamosa L*. leaves have IC_{50} values of 6 µg/mL, 9 µg/mL, and 8 µg/mL, accordingly. All samples of essential oil from *Annona Squamosa L*. leaves show increased absorbances with the increasing concentration of essential oils. The increased reading of absorbance indicates a higher reducing power in the essential oils.

[15] assessed the capacity of antioxidants in the fresh and dried leaf extracts of *Annona Squamosa L*. in two solvent systems which are ethanol and methanol solvents. The leaves were extracted using a maceration procedure for 24 hours, concentrated in a rotary evaporator at 40 °C, and undergoes the DPPH method. The fresh leaves of *Annona Squamosa L*. of ethanol and methanol extracts have IC_{50} values of 20.75 µg/mL and 27.35 µg/mL, respectively. The IC_{50} value for the ethanolic and methanolic extracts of the dried leaves of *Annona Squamosa L*. was 15.97 µg/mL and 13.61 µg/mL, accordingly.

[16] investigated the antioxidant activity of the methanol and hexane extracts of *Annona Squamosa L*. bark. The methanol and hexane extracts of *Annona Squamosa L*. bark was extracted using the Soxhlet extraction method by heating the solvent at 70 °C and 40 °C, respectively. The extracts were vaporised at 40 °C in a rotary evaporator and screened for antioxidant activity via the DPPH method. The IC_{50} value of *Annona Squamosa L*. bark for methanol and hexane extracts was 49.64 µg/mL and 64.01 µg/mL, accordingly.

[20] studies the antioxidant activity of *Annona Squamosa L*. leaves by extracting them using 96% ethanol solvent and undergoing the DPPH and ABTS assay for the determination of antioxidant activity. The ethanolic extract of *Annona Squamosa L*. leaves has an IC_{50} value of 132.96 ± 1.33 µg/mL and 64.74 ± 0.52 µg/mL for the DPPH and ABTS assay, proportionately.

[21] conducted an *in-vitro* study of the potential antioxidant activity in *Annona Squamosa L*. leaves. *Annona Squamosa L*. leaves were extracted into three different extracts which are aqueous, hexane, and methanol extracts by using water, hexane, and methanol, respectively. The extracts of *Annona Squamosa L*. leaves were concentrated to produce semisolid products which will be used in investigating the antioxidant activity of the aqueous, hexane, and methanol extracts of *Annona Squamosa L*. leaves using the DPPH free radical scavenging assay. Different concentrations (100 µg/mL to 1000 µg/mL) of all three extracts of *Annona Squamosa L*. seeds were used in obtaining the IC_{50} value. The IC_{50} value of aqueous, hexane, and methanol extracts for *Annona Squamosa L*. leaves was 112.35 ± 1.76 µg/mL, 84.67 ± 0.47 µg/mL, and 11.47 ± 0.22 µg/mL, respectively. The IC_{50} value of the methanol extract of *Annona Squamosa L*. leaves is the lowest compared to the aqueous and hexane extracts which show it has high potential antioxidant activity.

[22] investigated the antioxidant activity of rutin compound isolated from *Annona Squamosa L*. leaves via the DPPH and FRAP assay. The leaves of *Annona Squamosa L*. were extracted using the Soxhlet apparatus with 250 mL of 80% ethanol. The extract was filtered and concentrated until it reaches the volume of 10 mL and mixed with 25 mL of purified water. The mixture was extracted with 50 mL of petroleum ether and 50 mL of chloroform in triplicate for each solvent. The collected aqueous layer remained upright for 72 hours in cold conditions to separate a yellow precipitate from the solution. The yellow precipitate was filtered and washed with a mixture of a solution containing chloroform, ethyl acetate, and ethanol with a ratio of 50:25:25, respectively. The undissolved substance was dissolved in hot methanol and

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filtered. The filtrate was evaporated to obtain 110 mg of yellow powder. The IC_{50} value of rutin from *Annona Squamosa L*. leaves was 4.92 µg/mL for the DPPH method. In the FRAP assay, the reducing power of rutin isolated from *Annona Squamosa L*. leaves was increased with increasing the concentration as the concentration of rutin isolated was 100 µg/mL giving the absorbance of 0.908 nm.

Peel

[15] determined the antioxidant activity of the *Annona Squamosa L*. pericarp using a maceration process with two solvents; ethanol and methanol. The extraction process was done for 24 hours, and the samples were concentrated using a rotary evaporator. *Annona Squamosa L*. pericarp of ethanolic extract has an IC_{50} value of 76.47 µg/mL and a methanolic extract of 70.91 µg/mL in the DPPH assay.

[23] explored the activity of antioxidants in *Annona Squamosa L*. peels in ten different cultivars via the ABTS, FRAP, DPPH, and ORAC (oxygen radical absorbance activity) assays. The peel of *Annona Squamosa L*. peels were freeze-dried and extracted using 95% ethanol at 25 °C for 24 hours with continuous shaking at 120 rpm and the extracts were filtered. The ten different cultivars of *Annona Squamosa L*. include 'Fai Keaw', 'Fai Krung', 'Nhung Keaw', 'Nung Krung', 'Nung Thong', 'Pakchong KU-1', 'Pakchong KU-2', 'Petch Pakchong', 'Nhur Thong', and 'African Pride'. ABTS, FRAP, and ORAC assay was expressed in mmol of trolox/g dry sugar apple peel. The ABTS values for 'Fai Keaw', 'Fai Krung', 'Nhung Keaw', 'Nung Krung', 'Nung Thong', 'Pakchong KU-1', 'Pakchong KU-2', 'Petch Pakchong', 'Nhur Thong', and 'African Pride' were 1.57, 0.60, 1.30, 0.74, 0.95, 0.89, 1.04, 0.45, 1.28, and 1.08, respectively. The FRAP values for ten different cultivators were 'Fai Keaw' (0.43), 'Fai Krung' (0.12), 'Nhung Keaw' (0.34), 'Nung Krung' (0.20), 'Nung Thong' (0.23), 'Pakchong KU-1' (0.21), 'Pakchong KU-2' (0.24), 'Petch Pakchong' (0.11), 'Nhur Thong' (0.27), and 'African Pride' (0.22). The cultivator 'Fai Keaw', 'Fai Krung', 'Nhung Keaw', 'Nung Krung', 'Nung Thong', 'Pakchong KU-1', 'Pakchong KU-2', 'Petch Pakchong', 'Nhur Thong', and 'African Pride' have the ORAC values of 2.84, 2.02, 2.80, 2.60, 2.46, 2.22, 2.57, 1.83, 2.56, and 2.27. The EC_{50} value of *Annona Squamosa L*. peels for 'Fai Keaw', 'Fai Krung', 'Nhung Keaw', 'Nung Krung', 'Nung Thong', 'Pakchong KU-1', 'Pakchong KU-2', 'Petch Pakchong', 'Nhur Thong', and 'African Pride' was 0.42 mg/mL, 1.78 mg/mL, 0.50 mg/mL, 1.10 mg/mL, 0.73 mg/mL, 1.19 mg/mL, 0.85 mg/mL, 3.06 mg/mL, 0.71 mg/mL, and 0.90 mg/mL, accordingly.

[24] investigated the free radical scavenging activity by the DPPH and ABTS methods with the manipulation of different extraction times which is 30, 40, 50, and 60 minutes; the concentration of ethanol like 40, 50, 60, and 70 %; the ratio of solvent to solid such as 15:1, 20:1, 25:1, and 30:1; and the temperature of the extraction process with 40, 50, 60 and 70 °C. The peel of *Annona Squamosa L*. was extracted using the maceration extraction method in the manipulated condition. The ideal condition obtained from the extraction was 52% ethanol concentration and extraction times of 51 minutes at a temperature of 60 °C with a ratio of 26 mL/g. The optimum antioxidant activity obtained from the various extraction process was 536.64 µmol TE/g and 1310.83 µmol TE/g for DPPH and ABTS assay, respectively.

[25] studied chemical profiling and the antioxidant property of *Annona Squamosa L*. peel. The peel of *Annona Squamosa L*. was extracted using the freeze-dried method and macerated in a methanol solution. The methanolic extract of *Annona Squamosa L*. peel was evaporated in a rotary evaporator. The antioxidant activity for *Annona Squamosa L*.'s methanolic extract was done using the ABTS method. An amount of 10 µL of methanolic extract of *Annona Squamosa L*. peel has an antioxidant capacity of 55.23 ± 0.3 mmol TE/100 mg freeze-dried peel.

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Pulp

[14] assessed the total antioxidant capacity of the ripened and unripen pulp of *Annona Squamosa L*. using FRAP assay. *Annona Squamosa L*. pulp was carried out by homogenizing the pulp with a chilled 80% methanol for 5 minutes under chilled conditions and filtering using a Buchner funnel under vacuum conditions. The filtrate was marked up to the final volume of 50 mL with chilled 80% methanol. The total antioxidant capacity of *Annona Squamosa L*. for ripened and unripen pulp was 0.94 ± 0.01 mg TE/g DW and 6.71 ± 0.00 mg TE/g DW, respectively.

[15] conducted a study on the antioxidant activity of *Annona Squamosa L*. pulp via the DPPH method. The pulp of *Annona Squamosa L*. pulp was extracted using the maceration process for 24 hours in two different solvents which are ethanol and methanol. The samples were evaporated in a rotary evaporator at 40 °C. The ethanolic and methanolic extract of *Annona Squamosa L*. pulp has the IC_{50} value of 659.68 µg/mL and 871.33 µg/mL, respectively.

[26] investigated the potential antioxidant activity of *Annona Squamosa L*. pulp through five different methods which are DPPH, ABTS, Fe^{3+} reduction, 2-Deoxyribose (2-DR) protection, and *β*-carotene protection assays. The pulp of *Annona Squamosa L*. was lyophilised, and extracted in methanol (1:1 w/v) for 72 hours, filtered, and concentrated under reduced pressure at 50°C. The IC_{50} value of the *Annona Squamosa L*. pulp extract for DPPH, ABTS, Fe^{3+} reduction, 2-Deoxyribose (2-DR) protection, and *β*-carotene protection was 0.83 ± 0.02 mg/mL, 0.38 ± 0.02 mg/mL, 0.74 ± 0.05 mg/mL, 0.43 ± 0.16 mg/mL, and 1.36 ± 0.02 mg/mL, correspondingly.

Root

[14] conducted a study on the total antioxidant capacity of *Annona Squamosa L*. root by FRAP assay. The root of *Annona Squamosa L*. was extracted by air-dried the sample, and ground into powdered form, mixed with 5 mL of 80% methanol, vortexed for 15 minutes, heated for 40 minutes at 60 °C in a water bath and centrifuged at 4,000 rpm for 5 minutes. The supernatant was removed, and the sediment undergo an extraction procedure. The total antioxidant capacity of *Annona Squamosa L*. root was 63.75 ± 1.36 mg TE/g DW.

Seed

[27] conducted a study on the antioxidant enzyme activity by the DPPH method. The seeds of *Annona Squamosa L*. were extracted in 95% ethanol within the ratio of 1:2.5 for 24 hours in ambient temperature. The extract was filtered and concentrated using a rotary evaporator at 45 °C. The percentage of inhibition for *Annona Squamosa L*. seed extract was 98% of inhibition.

[9] determined the antioxidant activity of *Annona Squamosa L*. seed oil via DPPH and FRAP assay. The seed of *Annona Squamosa L*. was extracted by soaking the seeds in distilled n-hexane for 72 hours, filtered and evaporated in a rotary evaporator at the temperature of 40 °C to form a concentrated extract. The seed oil of *Annona Squamosa L*. was extracted using the Soxhlet extraction method and n-hexane was used as the solvent in the extraction process. The IC_{50} value for seed oil of *Annona Squamosa L*. in DPPH, and FRAP value were 1.33 ± 0.001 mg/mL, and 34.8 ± 0.01 mg AAE/g (mg Ascorbic Acid Equivalents/g), respectively.

[28] assessed the antioxidant effects of *Annona Squamosa L*. seeds in four different solvents which are petroleum ether, acetone, ethanol, and methanol. The extracts were concentrated using a rotary evaporator. The scavenging activity of *Annona Squamosa L*. seeds extracts were investigated in DPPH, superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and nitric oxide (NO) assays. *Annona Squamosa L*. seeds extract in the DPPH assay show that the ethanol extract has the EC_{50} value of 9.00 ±

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0.26 µg/mL, followed by methanol extract which has 9.80 ± 0.13 µg/mL, while the petroleum ether extract has 87.50 ± 0.24 µg/mL, and acetone extract was 93.20 ± 0.10 µg/mL. The EC_{50} value in the superoxide scavenging activity assay of *Annona Squamosa L*. seeds were 9.40 ± 0.13 µg/mL, 22.11 ± 0.32 µg/mL, 99.90 ± 0.22 µg/mL, and less than 400.00 ± 0.17 µg/mL for ethanol, methanol, acetone, and petroleum ether extracts, respectively. The ethanol, methanol, petroleum ether, and acetone extracts of *Annona Squamosa L*. seeds in hydrogen peroxide scavenging activity assay give the EC_{50} value of 10.80 ± 0.32 µg/mL, 14.50 ± 0.21µg/mL, 92.50 ± 0.21 µg/mL, and 98.10 ± 0.42 µg/mL, accordingly. The EC_{50} value of *Annona Squamosa L*. seeds in methanol, ethanol, petroleum ether, and acetone extracts via the nitric oxide scavenging activity assay, therefore, 39.50 ± 0.11 µg/mL, 47.50 ± 0.31 µg/mL, 88.50 ± 0.14 µg/mL, and 93.10 ± 0.28 µg/mL.

[14] analysed the total antioxidant capacity of *Annona Squamosa L*. seed was 6.43 ± 0.00 mg TE/g DW via the FRAP assay. The seed of *Annona Squamosa L*. was extracted by air-dried seeds, ground into powdered form, mixed in 5 mL of 80% methanol, vortexed for 15 minutes, heated at 60 °C in a water bath for 40 minutes and centrifuged at 4,000 rpm for 5 minutes. The supernatant was decanted in a 15 mL of centrifuge tube and the extraction procedure was repeated for the remaining sediment.

[26] studied the antioxidant activity of *Annona Squamosa L*. seeds via the DPPH, ABTS, Fe^{3+} reduction, 2-Deoxyribose (2-DR) protection, and *β*-carotene protection procedures. The seeds of *Annona Squamosa L*. were grounded into a fine powder and extracted using the Soxhlet extraction method with methanol as a solvent. The extract was vaporised under reduced pressure at 50 °C. The seeds extract of *Annona Squamosa L*. have an IC_{50} value of 0.36 ± 0.02 mg/mL, 0.14 ± 0.02 mg/mL, 0.57 ± 0.01 mg/mL, 0.41 ± 0.019 mg/mL, and 0.16 ± 0.03 mg/mL of DPPH, ABTS, Fe^{3+} reduction, 2-DR protection, and *β*-carotene protection, respectively.

[21] have investigated the potential of antioxidant activity for *Annona Squamosa L*. seeds. The seeds of *Annona Squamosa L*. were extracted using a maceration process with three different solvents; hexane, methanol, and water to obtain hexane, methanol, and aqueous extracts, respectively. All three extracts were concentrated using a water bath to acquire semisolid products. The hexane, methanol, and aqueous extracts of *Annona Squamosa L*. seeds were assessed for their antioxidant activity using the DPPH method. The IC_{50} value for the hexane extract of *Annona Squamosa L*. seeds was 115.45 ± 1.12 µg/mL while the methanolic extract of *Annona Squamosa L*. seeds was 110.00 ± 0.264 µg/mL. The aqueous extract of *Annona Squamosa L*. seeds were 75.57 ± 0.67 µg/mL.

[29] explored the potential antioxidant activity of *Annona Squamosa L*. seeds. *Annona Squamosa L*. seeds were extracted in distilled water (1:20 w/v) and heated for 30 minutes at 70 °C. The extract was centrifuged at 10,000 × g for 25 minutes, filtered, and assessed for antioxidant activities via DPPH and ABTS methods. In the DPPH method, the IC_{50} value of *Annona Squamosa L*. seeds extract was 7.88 ± 0.28 µg/mL. The IC_{50} value of *Annona Squamosa L*. seeds extract via the ABTS method was between the range of 15 to 20 µg/mL. The antioxidant properties of *Annona Squamosa L*. has been summarized in (Table 2).

Anti-Inflammatory Properties of *Annona Squamosa L.*

Bark

[30] investigated the anti-inflammatory activity of *Annona Squamosa L*. bark by the carrageenan-induced paw edema method. The bark of *Annona Squamosa L*. was extracted using petroleum ether in the Soxhlet extraction method with a temperature between the range of 40 to 60°C. The solvent was

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Table 2: Antioxidant Properties of *Annona Squamosa* L.

Type of Extract	Chemical Compound	Methodology	Results/Findings	References
BARK				
80% methanolic extract	—	FRAP assay	Total antioxidant capacity of 33.09 ± 0.54 mg TE/g DW	[14]
80% methanolic extract	—	DPPH assay	IC_{50} value of 38.49 µg/mL	[15]
80% ethanolic extract	—	DPPH assay	IC_{50} value of 55.77 µg/mL	
Hexane extract	—	DPPH assay	IC_{50} value of 132.23 µg/mL	[16]
Methanolic extract	—	DPPH assay	IC_{50} value of 70.55 µg/mL	
LEAVES				
Acetone extract	—	DPPH assay	IC_{50} value of 33.9 ± 4.8 µg/mL	[17]
		H2O2 assay	IC_{50} value of 516.7 ± 5.8 µg/mL	
		NO assay	IC_{50} value of 44 ± 5.7 µg/mL	
		Reducing power assay	Reducing power of 0.9_{50} µg/mL	
Methanolic extract	—	DPPH assay	IC_{50} value of 51 ± 1.6 µg/mL	
		H2O2 assay	IC_{50} value of 735 ± 49.5 µg/mL	
		NO assay	IC_{50} value of 12 ± 4.2 µg/mL	
		Reducing power assay	Reducing power of 0.975 µg/mL	
Aqueous extract	—	DPPH assay	IC_{50} value of 98.3 ± 0.4 µg/mL	
		H2O2 assay	IC_{50} value of 110 ± 14.1 µg/mL	
		NO assay	IC_{50} value of 81 ± 1.4 µg/mL	
		Reducing power assay	Reducing power of 0.984 µg/mL	

(*Contd.*)

Properties of *Annona Squamosa L*.

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Table 2: Antioxidant Properties of *Annona Squamosa* L. (*Contd.*)

Type of Extract	Chemical Compound	Methodology	Results/Findings	References
Acetone extract	—	DPPH assay	IC_{50} value of 396.43 ± 0.9 µg/mL	[18]
Chloroform extract	—		IC_{50} value of 234.69 ± 0.5 µg/mL	
Hexane extract	—		IC_{50} value of 438.79 ± 0.1 µg/mL	
Methanol extract	—		IC_{50} value of 96.09 ± 1.3 µg/mL	
Petroleum ether extract	—		IC_{50} value of 361.22 ± 0.7 µg/mL	
Aqueous extract	—		IC_{50} value of 148.09 ± 1.2 µg/mL	
80% methanolic extract	—	FRAP assay	Total antioxidant capacity of 53.39 ± 0.48 mg TE/g DW.	[14]
Essential oil	—	DPPH assay	IC_{50}values;—	
1st EO: 6 µg/mL; 2nd EO: 9 µg/mL; 3rd EO: 8 µg/mL	[19]			
	—	FRAP assay	Reducing power of essential oil increased with the increased concentration of essential oil.	
Ethanolic extract	—	DPPH assay	IC_{50}values:—	
Fresh leaves: 20.75 µg/mL; Dried leaves: 15.97 µg/mL	[15]			
Methanolic extract	—		IC_{50}values:—	
Fresh leaves: 27.35 µg/mL; Dried leaves: 13.61 µg/mL				
Hexane extract	—	DPPH assay	IC_{50} values of 64.01 µg/mL	[16]
Methanolic extract	—		IC_{50} values of 49.64 µg/mL	

(*Contd.*)

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Table 2: Antioxidant Properties of *Annona Squamosa* L. (*Contd.*)

Type of Extract	Chemical Compound	Methodology	Results/Findings	References
96% ethanolic extract	—	ABTS assay	IC_{50} values of 64.74 ± 0.52 µg/mL	[20]
		DPPH assay	IC_{50} values of 132.96 ± 1.33 µg/mL	
Aqueous extract	—	DPPH assay	IC_{50} values of 112.35 ± 1.76 µg/mL	[21]
Hexane extract	—		IC_{50} values of 84.67 ± 0.47 µg/mL	
Methanolic extract	—		IC_{50} values of 11.47 ± 0.22 µg/mL	
80% ethanolic extract	Rutin	FRAP assay	Reducing power of rutin increased with the increased concentration of rutin.	[22]
		DPPH assay	IC_{50} values of 4.92 µg/mL	
PEEL				
Ethanolic extract	—	DPPH assay	IC_{50} values of 76.47 µg/mL	[15]
Methanolic extract	—		IC_{50} values of 70.91 µg/mL	
95% ethanolic extract	—	ABTS assay	'Fai Keaw' cultivator has the highest value of 1.57 mmol of trolox/g dry sugar apple peel.	[23]
		FRAP assay	'Fai Keaw' cultivator has the highest value of 0.43 mmol of trolox/g dry sugar apple peel.	
		DPPH assay	'Fai Krung' cultivator has the highest EC_{50} values of 1.78 mg/mL	

(*Contd.*)

Properties of *Annona Squamosa L.*

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Table 2: Antioxidant Properties of *Annona Squamosa* L. (*Contd.*)

Type of Extract	Chemical Compound	Methodology	Results/Findings	References
		ORAC assay	'Fai Keaw' cultivator has the highest value of 2.84 mmol of trolox/g dry sugar apple peel.	
52% ethanolic extract	—	ABTS assay	Optimum antioxidant activity of 1310.83 µmol TE/g.	[24]
		DPPH assay	Optimum antioxidant activity of 536.64 µmol TE/g.	
Methanolic extract	—	ABTS assay	A 10 µL has an antioxidant capacity of 55.23 ± 0.3 mmol Trolox Equivalents/100 mg freeze-dried peel.	[25]
PULP				
80% methanolic extract	—	FRAP assay	Ripen pulp: 0.94 ± 0.01 mg TE/g DW; Unripen pulp: 6.71 ± 0.00 mg TE/g DW.	[14]
Ethanolic extract	—	DPPH assay	IC_{50} value of 659.68 µg/mL	[15]
Methanolic extract			IC_{50} value of 871.33 µg/mL	
Methanolic extract	—	ABTS assay	IC_{50} value of 0.38 ± 0.02 mg/mL	[26]
		DPPH assay	IC_{50} value of 0.83 ± 0.02 mg/mL	
		Fe3+ reduction assay	IC_{50} value of 0.74 ± 0.05 mg/mL	
		2-DR protection assay	IC_{50} value of 0.43 ± 0.16 mg/mL	
		β-carotene protection assay	IC_{50} value of 1.36 ± 0.02 mg/mL	

(*Contd.*)

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Table 2: Antioxidant Properties of *Annona Squamosa* L. (*Contd.*)

Type of Extract	Chemical Compound	Methodology	Results/Findings	References
ROOT				
80% methanolic extract	—	FRAP assay	Total antioxidant capacity of 63.75 ± 1.36 mg TE/g DW.	[14]
SEEDS				
95% ethanolic extract	—	DPPH assay	Percentage of inhibition of 98%.	[27]
N-hexane extract	—	FRAP assay	IC_{50} value of 34.8 ± 0.01 mg AAE/g.	[9]
		DPPH assay	IC_{50} value of 1.33 ± 0.001 mg/mL	
Acetone extract	—	DPPH assay	EC_{50} value of 93.20 ± 0.10 µg/mL	[28]
		$O_2^{\bullet}$– assay	EC_{50} value of 99.90 ± 0.22 µg/mL	
		H2O2 assay	EC_{50} value of 98.10 ± 0.42 µg/mL	
		NO assay	EC_{50} value of 93.10 ± 0.28 µg/mL	
Ethanolic extract	—	DPPH assay	EC_{50} value of 9.00 ± 0.26 µg/mL	
		$O_2^{\bullet}$– assay	EC_{50} value of 9.40 ± 0.13 µg/mL	
		H2O2 assay	EC_{50} value of 10.80 ± 0.32 µg/mL	
		NO assay	EC_{50} value of 47.50 ± 0.31 µg/mL	
Methanolic extract	—	DPPH assay	EC_{50} value of 9.80 ± 0.13 µg/mL	
		$O_2^{\bullet}$– assay	EC_{50} value of 22.11 ± 0.32 µg/mL	
		H2O2 assay	EC_{50} value of 14.50 ± 0.21µg/mL	
		NO assay	EC_{50} value of 39.50 ± 0.11 µg/mL	
Petroleum ether extract	—	DPPH assay	EC_{50} value of 87.50 ± 0.24 µg/mL	

(*Contd.*)

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Table 2: Antioxidant Properties of *Annona Squamosa* L. (*Contd.*)

Type of Extract	Chemical Compound	Methodology	Results/Findings	References
		$O_2^{\bullet}$– assay	EC_{50} value less than 400.00 ± 0.17 µg/mL	
		H2O2 assay	EC_{50} value of 92.50 ± 0.21 µg/mL	
		NO assay	EC_{50} value of 88.50 ± 0.14 µg/mL	
80% methanolic extract	—	FRAP assay	Total antioxidant capacity of 6.43 ± 0.00 mg TE/g DW.	[14]
Methanolic extract	—	ABTS assay	IC_{50} value of 0.14 ± 0.02 mg/mL	[26]
		DPPH assay	IC_{50} value of 0.36 ± 0.02 mg/mL	
		Fe3+ reduction assay	IC_{50} value of 0.57 ± 0.01 mg/mL	
		2-DR protection assay	IC_{50} value of 0.41 ± 0.019 mg/mL	
		β-carotene protection assay	IC_{50} value of 0.16 ± 0.03 mg/mL	
Aqueous extract	—	DPPH assay	IC_{50} value of 75.57 ± 0.67 µg/mL	[21]
Hexane extract			IC_{50} value of 115.45 ± 1.12 µg/mL	
Methanolic extract			IC_{50} value of 110.00 ± 0.264 µg/mL	
Aqueous extract	—	ABTS assay	IC_{50} value in the range between 15 to 20 µg/mL	[29]
		DPPH assay	IC_{50} value of 7.88 ± 0.28 µg/mL	

evaporated under vacuum conditions to acquire the petroleum ether extract and the extract undergo a purification process by the separation of saponified and unsaponified extract through the alcoholic-alkaline treatment. The unsaponified petroleum ether extract of *Annona Squamosa L*. bark undergoes a thin-layer chromatography (TLC) method to isolate the caryophyllene oxide. The Wistar rats (150 to 200 g) were selected and administered with 0.1 mL of 1% carrageenan suspension with 2% gum acacia in normal saline by injecting into the sub plantar of the right hind paw of rats after one hour of treatment administration. Aspirin with a dose of 100 mg/kg body weight acts as

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standard, the unsaponified petroleum ether extract with a dose of 50 mg/kg body weight, and the dose of 12.5 and 25 mg/kg body weight of caryophyllene oxide isolated from *Annona Squamosa L*. bark was administered orally. The volume of the paw was measured using a plethysmometer every hour for 3 hours after the injection of carrageenan suspension. The unsaponified petroleum ether extract (50 mg/kg body weight) and caryophyllene oxide (12.5 and 25 mg/kg body weight) show significant anti-inflammatory activity in the first and second hour on the inhibition of inflammation response.

[31] studied the anti-inflammatory activity of *Annona Squamosa L*. bark via the carrageenan-induced paw edema model. *Annona Squamosa L*. bark was extracted using the Soxhlet apparatus in a methanol solvent. The methanol solution was evaporated to obtain a crude methanol extract and applied to thin-layer chromatography to isolate caryophyllene oxide. Albino Wistar rats (150 to 200 g) were chosen, and acute inflammation was developed in the rats by injecting 0.1 mL of 1% carrageenan suspension with 2% gum acacia in a normal saline into the sub-plantar of the right hind paw of rats after one hour of orally administrating the test compounds. The test compounds consisted of the methanolic extract of *Annona Squamosa L*. bark with a dose of 50 mg/kg body weight, the dose of 12.5 and 25 mg/kg body weight of caryophyllene oxide, and aspirin with 100 mg/kg body weight that acts as a standard. The paw volume was measured every hour for 3 hours using a plethysmometer after the injection of carrageenan suspension. The methanolic extract (50 mg/kg body weight), and caryophyllene oxide (12.5 and 25 mg/kg body weight) exhibit significant anti-inflammatory activity by inhibiting the edema inflammation in the first and second hours.

Leaves

[32] investigated the anti-inflammatory activity of *Annona Squamosa L*. leaves by administrating formalin to induce edema in rats. The leaves of *Annona Squamosa L*. were extracted through the percolation extraction method using 70% ethanol until exhaustion. The extract was filtered and concentrated using a rotary evaporator in vacuum conditions at the temperature of 50 °C. The rats with weights in the range of 200 to 250 g were selected and undergo induction of inflammation at the right-hand paw of rats by injecting subcutaneously 0.1 mL of 6% formalin solution in normal saline. The thickness of the rats' paws was measured in mm using a vernier calliper after 4 hours of the injection procedure. Diclofenac sodium (Voltarin®) was administered orally with a dose of 30 mg/kg body weight and acts as a standard. A dose of 250 and 500 mg/kg body weight of the ethanolic extract of *Annona Squamosa L*. leaves were administered orally and 0.1 mL of 6% formalin in normal saline was injected subcutaneously at the right-hand paw of rats after thirty minutes of administrating the test sample. The thickness of the right-hand paw of rats was measured every hour using a vernier calliper in mm for 4 hours after administration of formalin. The ethanolic extract of *Annona Squamosa L*. leaves exhibited significant anti-inflammatory activity as there is a significant reduction in the thickness of the right-hand paw induced by the dose of 250 and 500 mg/kg body weight of *Annona Squamosa L*. leaves ethanolic extract.

[33] conducted a study on the ethanolic and aqueous extracts of *Annona Squamosa L*. on aluminium chloride-induced neuroinflammation in albino rats. The male albino rats (150 to 200 g) were selected and administered orally 50 mg/kg/day of aluminium chloride, $AlCl_3$ (pH 7.0) for two months to induce the neuroinflammation. A dose of 300 mg/kg/day of the ethanolic and aqueous extracts of *Annona Squamosa L*. leaves was administered orally to the rats for two months and the brain of rats was assessed in a biochemical analysis to determine the level of nitric oxide (NO), malondialdehyde (MDA), reduced glutathione

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(GSH), superoxide dismutase (SOD), acetylcholinesterase (AchE), brain-derived neurotrophic factor (BDNF), nuclear factor-?B (NF-?B), and caspase 3. Both the ethanolic and aqueous extracts of *Annona Squamosa L*. leaves show a significant decrease in MDA, NO, AchE activity, NF-?B, and caspase 3 while restoring GSH, SOD activity and BDNF close to the normal levels in the $AlCl_3$ rats. The ethanolic and aqueous extracts of *Annona Squamosa L*. leaves exhibit neuroprotective activity against the inflammation induced by aluminium chloride, $AlCl_3$.

[34] explored the effect of *Annona Squamosa L*. leaves against paracetamol-induced nephrotoxicity in rats. The leaves of *Annona Squamosa L*. were extracted in a Soxhlet extraction system for 18 hours using petroleum ether as the solvent and dried into powdered form. The dried powdered extract was extracted using the Soxhlet apparatus in an ethanol solution for 72 hours until exhaustion. The ethanolic extract of *Annona Squamosa L*. leaves undergoes experiments for *in vitro* human embryonic kidney-293, HEK-293 cells and *in vivo* paracetamol-induced nephrotoxicity in rats. The IC_{50} value for the ethanolic extract of *Annona Squamosa L*. leaves in the *in vitro* HEK-293 cells was 28.75 µg/mL which shows a significant development in cell growth and cytoprotective activity. *In vivo,* paracetamol-induced nephrotoxicity in rats was evaluated for the amount of blood urea nitrogen (BUN), creatinine, and uric acid as it showed reduction within the serum and the levels of glutathione (GSH), catalase (CAT), and superoxide dismutase (SOD) were increased in the kidney tissue from the treatment group receiving a dose of 200 and 400 mg/kg of ethanolic extract of *Annona Squamosa L*. leaves.

[35] analysed the expression and molecular involvement of NF-κB signalling biomarkers in HaCaT keratinocyte cells that are associated with psoriasis using sem-quantitative RT-PCR and report gene assays against *Annona Squamosa L*. leaves extract. *Annona Squamosa L*. leaves were extracted using the maceration process in ethanol solution (1:5 w/v) at room temperature for 48 hours on a shaking incubator at 120 rpm. The extract was filtered, concentrated using a MiVac Quattro concentrator at 45 °C, and dissolved in DMSO solution as a stock solution with a concentration of 100 mg/mL. The ethanolic extract of *Annona Squamosa L*. leaves exhibits a significant declining expression of CD40 and NF-κB1 and the capability of controlling the expression of NF-κB signalling biomarkers to give anti-psoriasis activity.

[36] assessed the anti-inflammatory activity of *Annona Squamosa L*. leaves by the carrageenan-induced paw edema model. The extraction of *Annona Squamosa L*. leaves were done in the ethanol solution using the Soxhlet extraction method for 24 hours and concentrated under reduced pressure conditions and temperatures of 50 to 60 °C to yield a solid extract. The Wister albino rats (150 to 180 g) were selected and administered orally with the test compounds of Indomethacin and ethanolic extract of *Annona Squamosa L*. leaves. After one hour of treatment, the rats were injected with 0.1 mL of 1% carrageenan suspension in normal saline to the sub-plantar left hind paws of rats to induce edema. The volume of the paw was measured for 5 hours at the first, third and fifth hour after injection of carrageenan suspension using a plethysmometer. A dose of 100 and 200 mg/kg of ethanolic extract of *Annona Squamosa L*. leaves administered orally shows a reduction in the inflammation induced with carrageenan suspension by 53% and 47%. *Annona Squamosa L*. gives significant anti-inflammatory activity against the carrageenan-induced rat paw edema method.

[37] evaluated the anti-inflammatory activity of *Annona Squamosa L*. leaves via the carrageenan-induced hind paw edema method. The extraction process for leaves of *Annona Squamosa L*. was done in a hot continuous extraction condition using ethanol as a solvent for 13 hours. The extract was evaporated in a water bath to obtain a concentrated extract. The adult male Wistar

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rats (150 to 200 g) were selected and administrated intraperitoneally with 10 mg/kg of aceclofenac sodium which acts as a standard, and 100 mg/kg of ethanolic extract of *Annona Squamosa L*. leaves. The rats were injected with 0.1 mL 1% w/v carrageenan suspension into the sub-plantar region of the right hind paw of rats after one hour of treatment. The volumes of the paw were measured in triplicate using a plethysmometer every hour for 3 hours. *Annona Squamosa L*. leaves ethanolic extract with a dose of 100 mg/kg exhibits a significant reduction and maximum inhibition by 47.16% of the carrageenan-induced right hind paw edema after two hours of treatment with the test compound.

Peel

[38] studied the anti-inflammatory effect of *Annona Squamosa L*. peel on Freund's Complete Adjuvant (FCA) induced rheumatoid arthritis in mice models. The peel of *Annona Squamosa L*. was extracted using a modified microwave machine with 60% ethanol and a ratio of 25:1 v/w for 5 minutes, microwave power of 214 W and the extract was filtered. The mice with a weight between 32 to 34 g were selected and treated with the test compounds starting on the seventh day (day 7). The mice were injected with a single dose of 0.1 mL of FCA and maintained for 12 days to establish rheumatoid arthritis. The peel extract of *Annona Squamosa L*. was administered orally to the FCA mice group with a dose of 200, 300, and 500 mg/kg/day for 10 weeks. The measurement of body weight, peripheral leukocytes concentration, ankle joint temperature and diameter (mm) was taken on the zeroth day (day 0) which is before the development of rheumatoid arthritis, after its development which is on the third, sixth, ninth, and twelfth day (day 3, 6, 9, and 12), and after the treatment with the test compounds on the fourth, sixth, eighth, and tenth week (week 4, 6, 8, and 10). The peel extract of *Annona Squamosa L*. at a dose of 400 mg/kg/day exhibits prevention of the inflammatory cell growth of rheumatoid arthritis. The body weight of mice increased to 38.00 g, the concentration of leukocytes reduced to 5.23 x 10^3 cells/mm^3 and the diameter of the ankle joint decreased to 3.95 mm. The histological analysis shows *Annona Squamosa L*. peel extract inhibits the immune cells from invading the joint substrate, the formation of fiber was reduced, and the cartilage structure of the synovial membrane was restored.

Rhizome

[36] assessed the anti-inflammatory activity of *Annona Squamosa L*. rhizome by the carrageenan-induced paw edema model. *Annona Squamosa L*. rhizome was extracted using the ethanol solution by the Soxhlet extraction method for 24 hours and concentrated under reduced pressure and temperatures within the range of 50 to 60 °C to yield a solid extract. The Wister albino rats (150 to 180 g) were selected and the treatment was administered orally with the test compounds of Indomethacin and ethanolic extract of *Annona Squamosa L*. rhizome. After one hour of treatment, the rats were injected into the sub-plantar left hind paws of rats with 0.1 mL of 1% carrageenan suspension in normal saline to induce edema. The paw volume was measured at the first, third and fifth hour after an injection of carrageenan suspension using a plethysmometer for 5 hours. The ethanolic extract of *Annona Squamosa L*. rhizome exhibits significant anti-inflammatory activity through the inhibition of the inflammation response induced by carrageenan.

Root

[39] analysed the anti-inflammatory activity of *Annona Squamosa L*. root via the carrageenan-induced hind paw edema model using two different extracts which are alcoholic and aqueous extracts. The alcoholic extract of *Annona Squamosa L*. root was extracted using the Soxhlet extraction method and concentrated by a Buchi rotary evaporator to gain a solid reddish-brown extract. The aqueous extract of *Annona*

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Squamosa L. root was extracted through the percolation method using cold water. The albino rats and Swiss albino mice were selected and given orally 100 mg/kg of diclofenac sodium, and both the alcoholic and aqueous extracts of *Annona Squamosa L*. root with a dose of 200 and 400 mg/kg body weight. After one hour of treatment administration, 0.1 mL of 1% carrageenan suspension was injected into the sub-plantar tissue of the right hind paw of rats. The volume of the paw was measured after 3 and 24 hours of administration of carrageenan suspension. The percentage of edema inhibition after 24 hours for the alcoholic extract of *Annona Squamosa L*. with the dose of 200 and 400 mg/kg body weight was 40% and 54%, respectively. The aqueous extract of *Annona Squamosa L*. with the dosing of 200 and 400 mg/kg body weight has the percentage of edema inhibition after 24 hours were 24% and 47%, accordingly.

Seed

[27] explored the impact of *Annona Squamosa L*. seeds ethanolic extract against inflammation in the kidney of rats induced by Ifosfamide. The extraction of *Annona Squamosa L*. seeds were extracted with 95% ethanol with a ratio of 1:2.5 for 24 hours. The extract was strained and evaporated using the rotary evaporator at 45 °C. *Wistar* albino male rats' weight 200 to 250 g were used and injected intraperitoneally with Ifosfamide only or a combination of *Annona Squamosa L*. seeds extract with Ifosfamide. The impact of *Annona Squamosa L*. seed extract against inflammation in the rat kidney induced by Ifosfamide was measured through the gene expression of iNOS and NF-κB, and a histopathological study of the kidney tissues. A dose of 50 mg/kg body weight of *Annona Squamosa L*. seeds extract shows an up-regulation of iNOS mRNA and a reduction in NF-κB mRNA in the rat kidney. The histopathological examination of *Annona Squamosa L*. seed extract exhibits an improvement in the glomerular and tubules in the kidney tissues.

[32] studied the anti-inflammatory activity of the ethanolic extract of *Annona Squamosa L*. seeds by inducing edema through the administration of formalin. The percolation extraction method was used to extract the seeds of *Annona Squamosa L*. several times until exhaustion by using 70% ethanol solution as the solvent. The extract was filtered and evaporated in a rotary evaporator to form a concentrated extract under a vacuum condition at 50 °C. Rats weighing between 200 to 250 g body weight were chosen and administered 0.1 mL of 6% formalin solution in normal saline by injecting subcutaneously at the right-hand paw of rats. The thickness of the right-hand paw of rats was measured after 4 hours of formalin administration using a vernier calliper and the measurement was recorded in mm. Diclofenac sodium (Voltarin®) acts as a standard in the test in a dosage of 30 mg/kg body weight. The ethanolic extract of *Annona Squamosa L*. seeds with a dose of 25 and 50 mg/kg body weight was administered orally and 0.1 mL of 6% formalin solution in normal saline was injected subcutaneously at the right-hand paw of rats after thirty minutes of administration of test compound. The measurement of thickness the right-hand paw of rats was measured every hour using a vernier calliper in mm for 4 hours. A dose of 25 and 50 mg/kg body weight of the ethanolic extract of *Annona Squamosa L*. seeds gives a significant anti-inflammatory activity as the thickness of the right-hand paw of rats was notably decreasing in a dose-related manner.

[40] reported a study on the anti-inflammatory activity of parallel synthesis of two cyclic peptides compounds isolated from *Annona Squamosa L.* seeds via anti-inflammatory screening for the evaluation of its inhibition on pro-inflammatory cytokines production using lipopolysaccharide (LPS) stimulated macrophage J774A.1 cells. The two cyclic peptides isolated from the seeds of *Annona Squamosa L*. cyclosquamosin D and Met-cherimolacyclopeptide B show significant anti-inflammatory activity in the suppression of IL-6 and TNF-α secretion.

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[41] assessed the anti-inflammatory activity of *Annona Squamosa L*. seeds through the carrageenan-induced hind paw edema method. The seeds of *Annona Squamosa L*. were extracted using the Soxhlet extraction method with the ethanol solution. The extract was concentrated using a rotary evaporator under reduced pressure to form a semi-solid. The albino rats with a weight between 150 to 200 g body weight were selected. Indomethacin acts as standard with the dosage of 10 mg/kg body weight and a dose of 100 mg/kg body weight of *Annona Squamosa L*. seeds extract was administered subcutaneously. The vehicle used was 5% w/v of acacia mucilage with a dose of 5 mL/kg. The test compound was administrated one hour prior to the experiment. The inflammation was induced through the single subplantar injection of 0.1 mL 1% w/v carrageenan solution in normal saline. The thickness of the left hind paw of rats was measured in triplicate before and after the injection procedure at every hour in triplicate for 8 hours. The dose of 100 mg/kg body weight of *Annona Squamosa L*. seeds extract shows significant anti-inflammatory activity through the inhibition of the edema. The seed extract of *Annona Squamosa L*. inhibits inflammation by about 36.33% in the carrageenan-induced hind paw edema method. The anti-inflammatory properties of *Annona Squamosa L*. has been listed in (Table 3).

Table 3: Anti-Inflammatory Properties of *Annona Squamosa L*.

Type of Extract	Chemical Compound Identified	Methodology	Results/Findings	References
BARK				
Unsaponified petroleum ether extract	Caryophyllene oxide	*In Vivo*: Carrageenan-induced paw edema	Oral administration of 12.5 and 25 mg/kg B.W. significantly inhibits the inflammation response in the 1st and 2nd hours.	[30]
	—		Oral administration of $_{50}$ mg/kg B.W. significantly inhibits the inflammation response in the 1st and 2nd hours.	
Methanolic extract	Caryophyllene oxide	*In Vivo*: Carrageenan-induced paw edema	Oral administration of 12.5 and 25 mg/kg B.W. significantly inhibits the edema inflammation in the 1st and 2nd hours.	[31]
	—		Oral administration of $_{50}$ mg/kg B.W. significantly inhibits the edema inflammation in the 1st and 2nd hours.	

(*Contd.*)

Properties of *Annona Squamosa L*.

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Table 3: Anti-Inflammatory Properties of *Annona Squamosa* L. (*Contd.*)

Type of Extract	Chemical Compound Identified	Methodology	Results/Findings	References
LEAVES				
70% ethanolic extract	—	*In Vivo*: Formalin-induced edema	Oral administration of 250 and 500 mg/kg B.W. significantly decreases the thickness of the right hind paw induced with formalin.	[32]
Aqueous extract	—	*In Vivo*: Aluminium chloride-induced neuroinflammation	Oral administration of 300 mg/kg B.W./day:— ↓ MDA, NO, AchE, NF-?B& caspase 3; ↑ GSH, SOD & BNDF.	[33]
Ethanolic extract				
Ethanolic extract	—	*In Vitro*: HEK-293 cell line	IC_{50} value of 28.75 µg/mL, significantly improves cell growth.	[34]
		In Vivo: Paracetamol-induced nephrotoxicity	Oral administration of 200 and 400 mg/kg B.W.:— ↓ BUN, creatinine & uric acid; ↑ GSH, CAT & SOD.	
Ethanolic extract	—	*In Vitro*: NF-κB signalling biomarkers in HaCaT keratinocyte cell line	Expression of CD40 and NF-κB1 significantly decreases and controls the expression of NF-κB signalling biomarkers.	[35]
Ethanolic extract	—	*In Vivo*: Carrageenan-induced paw edema	Oral administration of 100 and 200 mg/kg B.W. significantly reduces inflammation by 53% and 47%, respectively.	[36]

(*Contd.*)

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Table 3: Anti-Inflammatory Properties of *Annona Squamosa* L. (*Contd.*)

Type of Extract	Chemical Compound Identified	Methodology	Results/Findings	References
Ethanolic extract	—	*In Vivo*: Carrageenan-induced paw edema	Intraperitoneal administration of 100 mg/kg B.W. significantly inhibits inflammation by 47.16%.	[37]
PEEL				
60% ethanolic extract	—	*In Vivo*: Freund's Complete Adjuvant-induced rheumatoid arthritis	Oral administration of 400 mg/kg B.W./day significantly prevents the growth of rheumatoid arthritis inflammation cells.	[38]
RHIZOME				
Ethanolic extract	—	*In Vivo*: Carrageenan-induced paw edema	Oral administration of *Annona Squamosa L*. extract significantly inhibits inflammation response.	[36]
ROOT				
Aqueous extract	—	*In Vivo*: Carrageenan-induced paw edema	Oral administration of 200 and 400 mg/kg B.W. significantly reduces edema inflammation by 24% and 47%, respectively.	[39]
Alcoholic extract			Oral administration of 200 and 400 mg/kg B.W. significantly reduces edema inflammation by 40% and 54%, respectively.	
SEEDS				
95% ethanolic extract	—	*In Vivo*: Ifosfamide-induced nephrotoxocity	Oral administration of 50 mg/kg B.W.:— ↑ iNOS& NF-κB, improves glomerular and tubules in kidney tissues.	[27]

(*Contd.*)

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Table 3: Anti-Inflammatory Properties of *Annona Squamosa* L. (*Contd.*)

Type of Extract	Chemical Compound Identified	Methodology	Results/Findings	References
70% ethanolic extract	—	*In Vivo*: Formalin-induced edema	Oral administration of 25 and 50 mg/kg B.W. significantly reduces the thickness of the right hind paw induced with formalin.	[32]
—	Cyclosquamosin D Met-cherimolacyclopeptide B	*In Vitro*: Inhibition on pro-inflammatory cytokines production using lipopolysaccharide (LPS) stimulated macrophage J774A.1 cell	Both cyclic peptides significantly suppress the secretion of IL-6 and TNF-α.	[40]
Ethanolic extract	—	*In Vivo*: Carrageenan-induced paw edema	Subcutaneous administration of 100 mg/kg B.W. significantly inhibits edema inflammation by 36.33%.	[41]

Conclusion

Annona Squamosa L. possess significant antioxidant and anti-inflammatory activities in different parts of the fruit. The presence of distinct chemical constituents, phenolic compounds, flavonoid compounds, and other active chemical constituents might present a great opportunity for developing *Annona Squamosa L*. as a natural medicinal plant in the treatment of acute and chronic diseases. The assessment of different parts of *Annona Squamosa L*. for the antioxidant and anti-inflammatory activities highlights the importance of research focusing on the pharmacological and medicinal properties of *Annona Squamosa L*.

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Development of a Novel Co-processed Excipient Comprising of Microcrystalline Cellulose, Xylitol, Mannitol, and Crospovidone for Orally Disintegrating Tablets

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Abstract

Co-processing an excipient is categorized as a novel methodology applied in the preparation of tablet dosage forms. The main objective of this novel technique is to enhance the flow property of excipients commonly used in the production of tablets which correlates to the direct compression method of tablet production which is highly influenced by the powder characteristics such as dilution potential, compressibility or even flowability. Oral disintegrating tablets (ODT) is becoming a preferred choice in solid dosage form due to its benefits to the patient. It offers a rapid onset of action, improved bioavailability, ease of administration, and improvement in patient compliance to medication, ideal for individuals such as adolescents or elderly that have difficulty in swallowing medications. Although ODTs offers a myriad of merits, there are still some niches where further research may be conducted to improve the formulation, these includes optimizing stability condition, as well as improving mechanical strength of the formulation. The most common method to produce ODTs is through direct compression. Corresponding to the discussion points above, this study was carried out to formulate a novel co-processed excipient that consists of microcrystalline cellulose (MCC), xylitol, crospovidone, and mannitol as an orally disintegrating tablet dosage form. The study was conducted in 2 stages. The first stage involves developing 6 different formulations of ODTs that differs in the percentage of co-processed excipients such as crospovidone or croscarmellose sodium. Following Stage 1 will be the characterization tests. These includes pre-, and post- compression tests respectively to evaluate the standard of each formulation. Formulation 3 that consist of 72% MCC, 10% xylitol, 10% mannitol, and 8% crospovidone was selected as the optimum co-processed excipient formulation as it achieved fastest disintegration time in comparison to the remaining formulations and was tested with dissolution test to identify the efficiency of drug release. Besides, Formulation 3 also portrayed desired results in other evaluation tests such as friability test weight variation test and hardness test. Co-processed excipient that is characterized by an improve in functionality and disintegration process is beneficial in the application of oral disintegration tablets.

Keywords: Co-processed excipient; formulation; oral disintegrating tablets; optimization

Introduction

ODT as defined by the U.S. Food and Drug Administration (FDA) is a dosage form that can disintegrate within seconds in contact with saliva in the oral cavity (1).The aim of ODT primarily targets patients that have dysphagia specifically, geriatrics,

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pediatrics, bedridden, psychiatrics, and nauseated patients with the intention to reduce any possible risk of chocking. ODT formulations also provides an avoidance in first pass metabolism which subsequently enhances the bioavailability of the drug, reducing dosing frequency and side effect of respective medication. There are many formulation methods that may be used to produce an ODT and method has respective advantages and drawbacks, while the most used method will be direct compression as it has low handling cost, and comparatively simple stages involved with near to zero losses (2).

One of the main focuses in production of tablets will be the choice of excipients. Each excipient carries respective pros and cons and could play a variety of role in the formulation process. Excipients may be co-processed to enhance functionality of individual excipients and assist in overcoming straggles during the formulation process of ODTs. Co-processed excipients are described as a combination of two or more excipients which is developed to alter respective physical properties in a way that is unachievable with the conventional way such as mixing and without considerable changes in chemical property. This technique aims to improve the overall flow property of the excipients in comparison to when used individually, which usually gives an enhancement of the binding and blending property of the excipients during the production process. Hence, co-processed excipients play a significant role in the formulation process, making the production of ODTs significantly simpler, hence concurrently optimizes the benefits of ODT and attain the qualities of an optimum ODT formulation (3).The high demand of multifunctionality excipients calls out the need to develop a new excipient and this is achievable by either creation of a completely new excipient or by developing a co-processed excipient with the existing excipients. The latter is favored as it is comparably more cost effective. An example of multifunctional co-processed excipient is microcrystalline cellulose (MCC) and starch. MCC is a poor tablet disintegrant however, it has an exceptional compaction property, while starch is a common disintegrant with poor flow and compaction. This combination often results in brittle tablets, hence the formulation of co-processed MCC and starch synergists each other and produces a robust tablet with desired mechanical and dissolution properties (4).The main objective of this study is to identify the ideal composition of co-processed excipients comprised of xylitol, MCC, mannitol, crospovidone and croscarmellose sodium (CCS) to produce an optimum ODT which will be loaded with the drug memantine hydrochloride to test the dissolution efficacy of the drug.

Memantine hydrochloride is a subtype of glutamate receptor and categorized as an antagonist of N-Methyl-D-Aspartate (NMDA) receptor, and it is indicated for moderate to severe Alzheimer's Disease and other neurodegenerative diseases. Memantine hydrochloride is available, as capsules, tablet, and solution form and it is important to take the entire solid dosage form content as a whole for optimum efficiency. However, due to rapid deterioration of motor function, and presence of dementia in Alzheimer's Disease individuals, it is often a challenge for this population to consume medication. Hence the presence of oral disintegrating tablets (ODT) fits as a solution to cater this problem as it offers fast disintegration without usage of any water within 60 seconds in the oral cavity. Testing of the co-processed excipient formulated ODT with memantine hydrochloride brings multiple beneficial for future development and discovery. The benefits of co-processed excipients are displayed in which it plays a crucial role in the production process as well as in improving the ODT formulation characteristics.

Materials and Methods

Materials used for this study includes croscarmellose sodium and

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crospovidone purchased from Merck KGaA; magnesium stearate and sodium chloride from R&M Chemicals; mannitol from Systerm; microcrystalline cellulose from Daily Chem; xylitol from MH Food; and 0.1N hydrochloric acid from Chemiz.

Equipment utilized in this study include digital balance from Mettler Toledo; Dissolution Tester and UV Spectroscopy from AHS Laboratory Supplies; disintegration tester, friability tester, tap density tester, and hardness tester from Electrolab and multiple punch tablet press from Karnavati.

Formulation of Oral Disintegrating Tablets (ODTs) with Co-Processed Excipient

Table 1 shows the formulation design for the preparation of ODT. First, all ingredients are weighed and grounded into fine powder using separate pestle and mortar. Next, suitable quantity of water, only enough to dissolve both excipients was added to xylitol and mannitol respectively to dissolve them. The water added should be less than half the amount of MCC to prevent the resulting mass from being excessively wet. Xylitol and mannitol were combined to form a granulating fluid and the combination was added to MCC, memantine hydrochloride, and crospovidone or CCS. A No. 12- sized mesh was used to sieve the resulting wet mass to obtain granules which were then allowed to dry for two hours within the incubator at a temperature not exceeding 50°C. The granules were subsequently passed through a No. 20-sized mesh after drying and finally subjected to compression into ODTs with the multiple punch tablet press.

Evaluation of Blend

Angle of Response

A method known as fixed funnel was applied in the following test. Granular materials are poured at a specific height from a funnel on to the base and stopped when it reaches a particular width or heigh. Next, information regarding conical shape's radius and maximum height were obtained.

Angle of repose was calculated with the following formula (5).

$Tan\theta = h/r$

h = Height of the cone

r = Radius of the cone

Compressibility Index and Hausner Ratio

Measurements regarding the powder's initial apparent volume (Vo) and the final tapped volume (Vf) after tapping the material until maximum volume change were obtained. The following formulas were applied to identify the compressibility index and Hausner ratio:

Compressibility Index (%):
100 × (VO - Vf)/VO
Hausner Ratio: Vo/Vf

Evaluation of Oral Disintegrating Tablets
Hardness Test

Hardness test was conducted by selecting ten blank tablets from each formulation randomly and subject the tablets to testing through the hardness tester.

Table 1: Formulation of Orally Disintegrating Tablets with Co-Processed Excipients

Excipient	F1	F2	F3	F4	F5	F6
MCC	77%	75%	72%	77%	75%	72%
Crospovidone	3%	5%	8%	-	-	-
CCS	-	-	-	3%	5%	8%
Xylitol	10%	10%	10%	10%	10%	10%
Mannitol	10%	10%	10%	10%	10%	10%

CCS = croscarmellose sodium.
MCC = microcrystalline cellulose

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Weight Variation

Twenty tablets from each formulation were selected randomly and weighed. The mean was identified, and all tablets weight are to be within the calculated upper limit and lower limit range. The following formula was applied in calculating the weight variation.

Weight Variation (%) =
[(Individual weight – Average weight)/ Average weight] × 100%

Friability Test

Roche friability tester was used to identify the friability of twenty randomly chosen tablets with a speed of 25rpm. The weight of the tablets before the test and after the test were identified to calculate the loss limit of each formulation. The following formula was used to determine the percentage of friability:

Friability (%) = [(W1 – W2) × 100] / W1
W1= Total weight of tablet before test
W2= Total weight of tablet after test

Disintegration Test

Disintegration test was conducted by randomly selecting six tablets from each formulation and place them in the six tubes of the disintegration time tester basket. The basket rack was positioned in a 1-L beaker container containing distilled water as the disintegration medium and maintained at 37°C which portrayed the human normal body temperature. The time for all six samples in the basket to distort in shape was observed and collected.

Selection of Optimum Formulation

The formulation that comprises of the fastest disintegration time with dominant pre- and post- compression test results is identified as the ideal formulation of memantine hydrochloride ODT. To achieve a complete formulation, the optimal formulation was subjected for dissolution test to identify the drug-release characteristics and consistency of the active component.

Dissolution Test

The test was conducted USP Apparatus 1 at 100rpm and 37°C. Six randomly selected tablets from the optimum formulation were dissolved in 900mL of 0.1N hydrochloric acid and 2g/L of sodium chloride in water. The solution is pH – adjusted with HCL to a pH of 1.2. 10mL of the sample was obtained at 10, 20, and 30 minutes respectively.

Results and Discussion

Evaluation of Blend

Angle of repose

Based on Figure 1, all formulations subjected to angle of repose test displayed excellent flowability which ranges within 25° to 30°. Among all formulations, Formulation 4 portrayed the best flowability as it has the lowest angle of repose (26.6°). Formulation 4 consists of 10% xylitol and mannitol, 77% of MCC, 3% of CCS, and 10mg of memantine hydrochloride. On the other hand, Formulation 1 depicted the highest angle of response, despite interpretated as having excellent flowability.

Angle of repose test is tightly correlated with the flow characteristics and stability of the granules produced (6). Excellent angle of repose suggests that aid may not be needed for the formulation process. The improvement in powder flow is due to increased particle size or spherical shape of the granules that occurred during co-processing. Hence, modification of particle size has a great impact on the flowability and compressibility of the excipients (7).

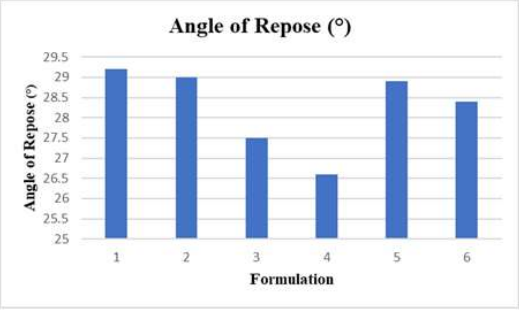

Figure 1: Results of Angle of Repose

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Compressibility Index and Hausner Ratio

According to Figures 2 and Figure 3 displayed in the appendix session, all formulations have good compressibility and flowability as they are all within a range of 11% to 15% and also within a Hausner ratio ranging from 1.12 to 1.18. Among all formulations, Formulation 4 displayed explicit flowability as it has the lowest Compressibility Index of 12.9% and Hausner ratio of 1.13. This formulation consists of 10% xylitol and mannitol, 72% of MCC, 8% of Crospovidone, as well as 10% of memantine hydrochloride.

In relation to the Compressibility Index and Hausner ratio results, it shows that the co-processed excipients lead to an improvement in terms of compressibility, which supports one of the studies reported Ludipress, a co-processed excipient has better compressibility in comparison with physical mixtures of their constituent excipient (3).

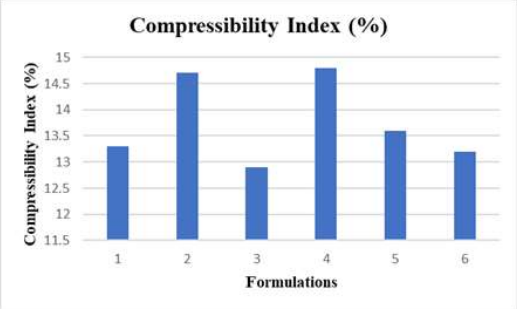


Figure 2: Results of Compressibility Index

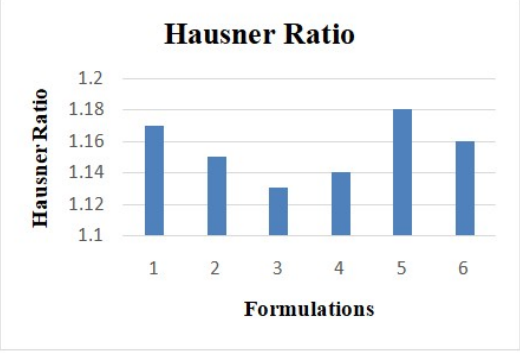


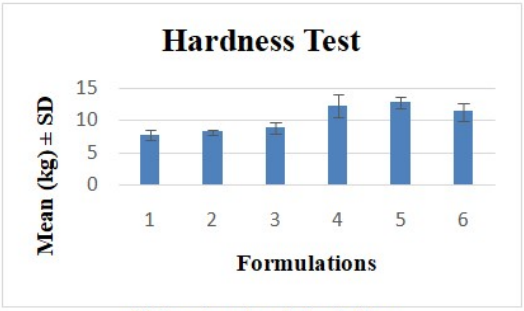
Figure 3: Results of Hausner Ratio

Several criteria are to be adhered to obtain a satisfactory result on the Compressibility Index and Hausner ratio. The amount of water added into the formulation during wet granulation process was increased in comparison to the amount mentioned in the reference article, however, the temperature and drying time was maintained as suggested to increase the moisture content of the granules which will ultimately lead to an improvement of flowability and compressibility since a large percentage of formulations consisted of MCC.

It was shown in research that the moisture content of MCC greatly affects the compaction, tensile, and viscoelastic properties of material. Moisture content in the pores of MCC serves as an internal lubricant and is important in lowering frictional forces, promoting slippage and plastic flow. The lubricant characteristics of water may further lessen variation in tablet weight by enhancing the passage of the compression force through the compact and reducing the adherence of the tablet weight. Generally, when the moisture content of MCC increases, relative compaction pressure required for formation of particular porosity reduces (8).

Evaluation of Oral Disintegrating Tablets
Hardness Test

Figure 4 shows the test of normality from Kruskal-Wallis test. Based on the obtained results, $p<0.001$ ($p<0.05$). A p value



SD = standard deviation

Figure 4: Results of hardness test

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less than 0.05 indicates that there was a statistically significant difference in hardness between the different formulations of ODTs containing memantine hydrochloride.

The results from Bonferroni post-hoc test shows that there was significant difference between Formulation 1, and Formulation 4,5,6; between Formulation 2, and Formulation 4,5,6; and between Formulation 3, and Formulation 4,5,6.

According to the United States Pharmacopoeia (USP), a satisfactory tablet hardness range from 5kg to 10kg. Based on the reference, only tablets from Formulation 1,2, and 3 passed the hardness test (9). Hardness from Formulation 4,5, and 6 exceeded the limit of 10kg and is not desired for ODT formulations. The difference between Formulation 1,2 and 3 with Formulation 4,5, and 6, were the super-disintegrants used. Formulation 1,2, and 3 contained Crospovidone, while Formulation 4,5, and 6 contained CCS which had variation in terms of porosity and might contribute to the difference in tablet hardness.

A study by Fathollahi et.al (2020) stated that CCS has high compressibility. The property of high-compressibility materials include that they are considerably densified upon pressure without significant elastic recovering. This indicates that the internal voids of highly compressible materials will be permanently destroyed as a result of rearrangement and shear when in contact with high pressure (10). Hence, this is why the type of super disintegrant may have an impact on the hardness, friability, as well as disintegration, absorption, and dissolution of the drug (11).

Weight Variation

Figure 5 shows the test of normality from Kruskal-Wallis test. Based on the obtained results, p=0.463 (p<0.05). A p value more than 0.05 indicates that there is no statistically significant difference in weight variation between the different formulations of ODTs containing memantine hydrochloride.

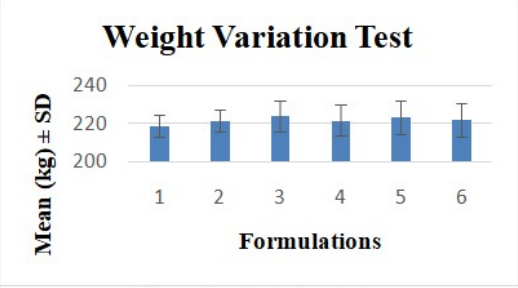

Figure 5: Results of Weight Variation Test

The formulated weight of memantine hydrochloride ODT in this study was 300mg. According to United States Pharmacopoeia (USP) standards, for tablets weighing between 130mg to 324mg, the weight variation readings of the tablets should be within a percentage deviation of ± 7.5%. Table 4 shows that all formulations are within the upper and lower limit of ± 7.5% and thereby achieved uniformity in weight. The tableting process was done manually where each blend was carefully weighed to 300mg before pouring the blend into the die. This is important to ensure the uniformity of each batch, that each batch of drug contains equal amount of API, and that ultimately the end users receive the correct dosage of medication (12).

Friability Test

The acceptance loss limit for friability test is that it should not exceed one percent. All formulations passed the friability test and were within a loss limit range of 0.22% to 0.24%. Friability is a crucial criteria as easily damaged tablets would lead to variations in weight, and uniformity of dosing of the API. It would be a problem for storage and transport of product as well. It is important to conduct this test to ensure strength and hardness of the tablets to conclude that all tablets are capable of withstanding abrasion during handling, packaging, or delivery of the product (13).

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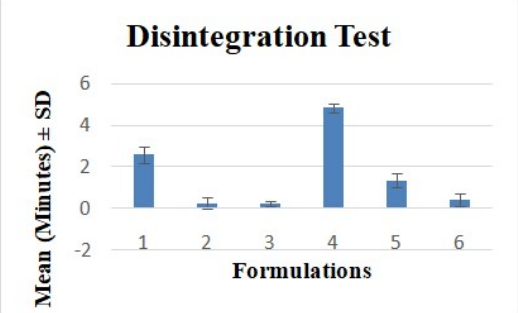
Disintegration Test

Figure 6 shows the test of normality from Kruskal-Wallis test. Based on the obtained results, $p<0.001$ ($p<0.05$). A p value less than 0.05 indicates that there is a statistically significant difference in disintegration profile between the different formulations of ODTs containing memantine hydrochloride.

The results from Bonferroni post-hoc test shows that there were differences between Formulation 3 and the remaining Formulations, except for Formulation 6; between Formulation 6, and Formulation 1, 4; between Formulation 2 and 4; and lastly between Formulation 5 and 1.

The ideal disintegration time for ODT is within 3 minutes (14).The results in Table 6 shows that all formulations meet the requirement for disintegration time and Formulation 3 stands the fastest disintegration time as it contains highest percentage of super disintegrant-crospovidone. Formulation 6 portrayed slower disintegration time in comparison to Formulation 3 as the super disintegrant used in this formulation is CCS (15). CCS has the tendency to form a viscous gel layer and prevent the disintegration medium from penetrating farther opposing the disintegration weight of the tablet. It is important to correlate the hardness result of Formulation 6 with Formulation 3 as well. Formulation 6 has a significantly higher hardness which will impact on the disintegration time as well since they are directly correlated (16). Finally, the poring of CCS differs with crospovidone as well. When hardness increases, pore distance reduces, which subsequently will lead to reduction in water permeability hence influencing the disintegration time of the tablets (17).



SD = standard deviation

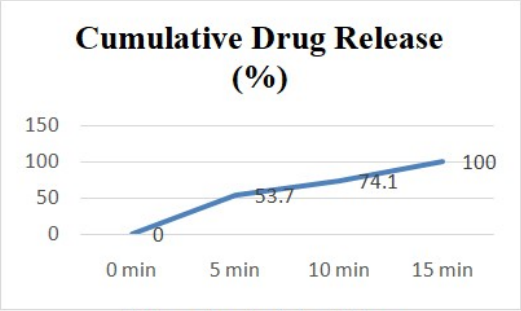
Figure 6: Results of Disintegration Test

Selection of Optimum Formulation

Conclusively, Formulation 3 which consists of 10% xylitol and mannitol, 72% MCC, 8% crospovidone, and 10mg memantine hydrochloride was chosen as the optimum formulation for the manufacturing of memantine hydrochloride ODT (18). This formulation stands out as it acquires the fastest disintegration time, with acceptable level of hardness, weight variation, and friability. In additionally, it also exhibits excellent flowability and good compressibility (19).

Dissolution Test

Figure 7 shows a line graph of the cumulative drug release profile of an optimum formulation of ODT containing memantine hydrochloride. The results shows that the drug content in the formulation was released slow and steadily reached full release profile at the 15th minute of the test. According to FDA, a minimum requirement of 85% API release is to be released within 30 minutes of dissolution test, Hence, this formulation of memantine hydrochloride ODT meets the specifications of the dissolution test (20).



SD = standard deviation

Figure 7: Dissolution profile of orally disintegrating tablets for formulation 3

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Conclusion

In conclusion, a co-processed excipient provides an improved functionality overall and has can be a potential trend in the pharmaceutical industry. In this study the four ingredients involved in the formulation of the co-processed excipients were xylitol, mannitol, MCC, and crospovidone or CCS. Ultimately, 10% xylitol, 10% mannitol, 72% MCC, and 8% Crospovidone were found to be the optimum composition as co-processed excipient and it shows acceptable results for all pre- and post-compression tests, displaying excellent disintegration time which is desired in an ODT formulation. Extension of study regarding taste masking of ODTs are highly recommended to expend the unlimited potential of discovery in the pharmaceutical field.

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Investigation on Flavanoid Extract From *Annona Squamosa L*. (Sugar Apple) Fruit For Potential Anti-Gout Property: *In Vitro* Studies

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Abstract

Introduction: *Annona squamosa L*. (Sugar Apple) belongs to the Annonaceae family. Different parts of *Annona squamosa L*. have various medicinal properties such as anti-inflammatory, antioxidant, antidiabetic, antimicrobial, antiulcer,and analgesic activity. Objective(s): To determine the antioxidant, anti-inflammatory and anti-gout properties of flavonoid extract of *Annona squamosa L*. fruit in the *in-vitro* and *in-vivo* studies. Methodologies: *Annona squamosa L*. fruit was extracted by maceration process using 70% ethanol solution and evaporated at 40°C. The ethanolic extract of *Annona squamosa L*. fruit was screened for the chemical tests of amino acids, carbohydrates, alkaloids, terpenoids, flavonoids, phenols, and tannins. The antioxidant activity of *Annona squamosa L*. fruit extract undergoes the total phenolic compound (TPC), ferric reducing ability of plasma (FRAP), and 2,2-diphenyl-1-picrylhydrazyl (DPPH) assays. Xanthine oxidase inhibitory (XOI) assay used for anti-gout property. Results: A 190 g of *Annona squamosa L*. fruit obtains 48.60 g of ethanolic extract. Chemical tests for carbohydrates, amino acids, flavonoids, tannins, and terpenoids have positive results while chemical tests for alkaloids, and phenols have negative results. The total phenolic content (TPC) is 8.106 mg GAE/g of *Annona squamosa L*. fruit.The EC_1 value for the FRAP assay of *Annona squamosa L*. fruit is 2.1307 mg/mL. The IC_{50} values for DPPH and XOI assays of *Annona squamosa L*. fruit are 4.1154 mg/mL and 4.4745 mg/mL, respectively. *Annona squamosa L*. fruit extract shows an increase in antioxidant activities and xanthine oxidase inhibition in a concentration-dependent manner. Conclusion: *Annona squamosa L.*fruit has the potential to be a source of antioxidant, anti-inflammatory, and anti-gout properties.

Keywords: *Annona squamosa L*., Sugar Apple, Antioxidant, Anti-inflammatory, Anti-gout

Introduction

Annona squamosa L. is a multipurpose plant that has several medicinal properties such as anti-inflammatory, antioxidant, antimicrobial, cytotoxic, antiulcer, hepatoprotective, antidiabetic, antilipidemic, antitumor, vasorelaxant, anthelmintic, genotoxic, and analgesic activity (1; 2). The medicinal properties involve bioactive compounds in the different parts of the *Annona squamosa L.* plant as it is used to treat ailments and human diseases. *Annona squamosa L.* is also used traditionally in treating epilepsy, constipation, haemorrhage, dysentery, fever, ulcer, worm infection, and cardiac complications (2).

Gout is the most common inflammatory disease due to hyperuricemia. Hyperuricemia is an elevation in the levelof the serum uric acid in the human body where it reaches a saturated level of 6.8 mg/dL at 37°C and pH 7, forming an inflammatory

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monosodium urate crystal (MSU) in the joints and synovium. Male and female patients with serum uric acid levels higher than 7 mg/dL and 6 mg/dL, respectively are categorised as hyperuricemia(3; 4).

In recent decades, the prevalence of gouty arthritis has been increasing worldwide due to the levitation of risk factors related with the disease, particularly hyperuricemia. Gout and hyperuricemia lead to the levitation of inflammation in the body, resulting in a high risk of complications such as cardiovascular diseases (5). A few of the risk factors of gout include sex, genetic variations, obesity, insulin resistance, medications, and kidney diseases related to the reduction of urate in renal clearance (6; 7).

Nowadays, there are a lot of drugs that have been discovered, developed, and suggested to have the ability to the treatment of gout. Fruits containing anti-inflammatory and anti-hyperuricemia activities might have potential benefits in gout treatment (7). This prompts the concern of discovering natural products that have the potential to treat gouty arthritis. *Annona squamosa L*. has been known as a folk medicine for its antioxidant and anti-inflammatory properties. Hence the study was undertaken to determine the antioxidant, anti-inflammatory and anti-gout properties of *Annona squamosa L*. fruit extract.

Materials and Methods

Chemicals

The chemicals used for this study included, 70% ethanol, Benedict's reagent, Millon's reagent, Mayer's reagent, chloroform, sulphuric acid (H_2SO_4),gallic acid, Folin-Ciocalteu reagent, sodium carbonate (Na_2CO_3), ascorbic acid, potassium ferricyanide ($K_3[Fe(CN)_6]$), trichloroacetic acid, ferric chloride ($FeCl_3$), disodium hydrogen phosphate (Na_2HPO_4), sodium dihydrogen phosphate (NaH_2PO_4), *2,2-diphenyl-1-picrylhydrazyl* (DPPH), dimethyl sulfoxide (DMSO), allopurinol, xanthine oxidase, xanthine, hydrochloric acid (HCl), sodium hydroxide (NaOH).

Sources of Annona squamosa L.

The identified *Annona squamosa L*. fruit was confirmed and conducted by a resident botanist at Mini Herbarium, Institute of Bioscience, Universiti Putra Malaysia (UPM), Serdang, Selangor with a voucher no of KM 0091/23.

Preparation of Annona squamosa L. Fruit Extract

A 5 kg of *Annona squamosa L*. fruit was dried in an oven at 50°C for 3 days and ground into a coarse powder. *Annona squamosa L*. fruit extract was prepared by maceration extraction method. The conical flask was filled with 70% ethanol following an extraction fraction ratio of 1:10 (fruit powder: solvent). The maceration process was done for 72 hours at ambient temperature with continuous swirling using an orbital shaker for thorough extraction. *Annona squamosa L*. fruitextract was filtered, evaporated using a rotary vacuum at 45°C evaporator under reduced pressure, and lyophilized to concentrate the extract. *Annona squamosa L*. fruit extract was kept in an airtight container ata temperature of–4°C(8).

Phytochemical Screening

The phytochemical screening was conducted on *Annona squamosa L*. fruit extract by identifying the presence of carbohydrates, alkaloids, flavonoids, amino acids, phenols, tannins, and terpenoids using chemical tests of Benedict's test Mayer's test, alkaline reagent test (9), Millon's test (10), ferric chloride test, ferric chloride-potassium ferricyanide test, and Salkowski's test (11), respectively.

Total Phenolic Content (TPC)

A 1 mL of *Annona squamosa L*. fruit extract is taken for 1 mL and added into a test tube. Next, the Folin-Ciocalteu reagent (FCR) solution and 10% of sodium carbonate (Na_2CO_3) solution are added into the test tube, wrapped with aluminium foil, and heated at 50°C for 5 minutes. The solution mixture is incubated for 10 minutes in a dark

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environment and the absorbance is measured at the wavelength of 415 nm. The blank solution contains the FCR solution and the Na_2CO_3 solution. The standard solution used in the determining the total phenolic content of *Annona squamosa L*. fruit extract is gallic acid. The procedure is carried out in triplicate. TPC of *Annona squamosa L*. fruit extract is expressed in mg gallic acid equivalent (GAE)/g of *Annona squamosa L*. fruit powder (8).

Ferric Reducing Antioxidant Power Assay (FRAP)

The ferric-reducing antioxidant power assay is done to determine the antioxidant activity of *Annona squamosa L*. fruit extract. Different concentrations of *Annona squamosa L*. fruit extract and ascorbic acid are prepared. Ascorbic acid is the reference standard. A 2.5 mL of 0.2 M phosphate buffer (PB) with pH 6.6 and 2.5 mL of 1% $K_3[Fe(CN)_6]$ are added into the test tube and incubated at 50°C for 20 minutes. Next, 2.5 mL of 10% trichloroacetic acid is added and centrifuged for 10 minutes at 3000 rpm. A 2.5 mLaliquot of supernatant from each test tube was mixed with 2.5 mL of distilled water and 0.5 mL of 0.1% $FeCl_3$. The blank solution contains PB, 1% $K_3[Fe(CN)_6]$, 10% trichloroacetic acid, 0.1% $FeCl_3$, and distilled water. The absorbance value was measured at 700 nm(12).A standard curve of 0.1 to 2.0 mM ferrous sulphate heptahydrate was constructed and expressed as mM Fe(II) per gram of dry-weight plant. EC_1 value of *Annona squamosa L*. fruit extract and ascorbic acid were evaluated based on the reading of absorbances equivalent to the theoretical absorbance value of 1 mM of Fe(II) concentration using the corresponding regression equation (13).

2,2-diphenyl-1-picrylhydrazyl (DPPH)

The antioxidant activity of *Annona squamosa L*. fruit extract is evaluated using the DPPH method. A reaction mixture consisting of *Annona squamosa L*. fruit extract and the DPPH reagent with different concentrations is prepared and incubated in a dark environment. The absorbance of the reaction mixture is measured at the wavelength of 517 nm. The process is performed in triplicate. The reference standard used in the DPPH method is ascorbic acid. The DPPH solution is prepared by dissolving the DPPH powder in the ethanol solution. The DPPH solution and the ethanol solution are used as control and blank, respectively in the procedure. The percentage of the scavenging effect of the sample is calculated using the formula;

$$\text{Scavenging effect (\%)} = (1 - \alpha/\beta) \times 100\%$$

Whereby α is the absorbance of the extractand β is the absorbance of the control. The results are expressed as mean ± SD. IC_{50} value (mg/mL) is the total antioxidant required to reduce the initial DPPH free radicals by 50% and derived from the graph of the percentage of scavenging activity plotted against the various concentrations of *Annona squamosa L*. fruit extract and ascorbic acid, respectively (13).

Xanthine Oxidase Inhibitory Assay (XOI)

The xanthine oxidase activity of *Annona squamosa L*. fruit extract is determined by calculating the formation of uric acid from xanthine. Allopurinol is used as the reference standard in the experiment. *Annona squamosa L*. fruit extract and allopurinol are dissolved and diluted in distilled water and DMSO, respectively to prepare a range of concentrations. A 0.1 mL sample with 1.9 mL of 50 mM PB with pH 7.5 and 0.1 units/mL xanthine oxidase enzyme is pre-incubated at 37°C for 15 minutes.

Next, 0.15 mM xanthine (1 mL) is added and incubated at 37°C for 30 minutes. Next, 0.5 M HCl is added to halt the reaction and the absorbance is measuredat the wavelength of 290 nm. The blank solution and the control used in the experiment are buffers and a solution consisting of xanthine and xanthine oxidase. The percentage of the xanthine oxidase inhibition

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activity (%) is calculated based on the given formula;

Inhibition activity (%) = (1 - α/ β) x 100%

Whereby, α is the absorbance of the extractand β is the absorbance of the control. The inhibition concentration (IC_{50}) that is required to inhibit 50% of the uric acid formation *Annona squamosa L*. fruit extract and allopurinol is evaluated from the standard curve of the inhibition of xanthine oxidase activity (13).

Statistical Analysis

For TPC, FRAP, DPPH, and XOI assays, the results were expressed in mean ± SD(13).

Results and Discussion

Percentage Yield of Annona squamosa L. fruit extract

The percentage yield of 70% ethanolic extraction was 25.58 %. A 190 g of *Annona squamosa L*. fruit powder (Figure 1) extracted in 70% ethanol solution obtained 48.60 g of *Annona squamosa L*. fruit extract and has the appearance of a dark, brown-coloured viscous and semi-solid form as shown in (Figure 2).

Phytochemical Screening

Phytochemical screening was done for *Annona squamosa L*. fruit extract. The chemical tests include identifying the presence of chemical constituents such as carbohydrates, amino acids, alkaloids, flavonoids, phenols, tannins, and terpenoids.

Figure 1: *Annona squamosa L*. Fruit Powder

The chemical test for carbohydrates, amino acids, flavonoids, tannins, and terpenoids has positive results while the chemical test for alkaloids, and phenols has negative results as shown in (Table 1).

Total Phenolic Content (TPC)

The total phenolic content of *Annona squamosa L*. fruit extract is 8.106 mg GAE/g of *Annona squamosa L*. fruit extract. The total phenolic content of *Annona squamosa L*. fruit extract was calculated based on the equation derived from the equationof standard curve for gallic acid which is $y = 0.301x + 0.238$ with the R^2 value of 0.902 as shown in (Figure 3). The presence of phenolic compounds in *Annona squamosa L*. fruit extract gives a potential antioxidant activity.

Based on the previous study, the total phenolic content of ethanol extraction was 13.53 mg GAE/g of *Annona squamosa L*. fruit extract. The plant's

Figure 2: *Annona squamosa L*. Fruit Extract

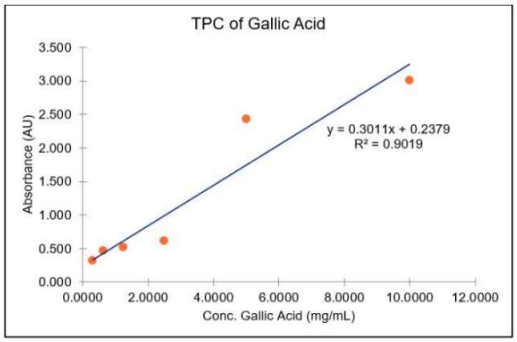

Figure 3: Total Phenolic Content of Gallic Acid

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Table 1: Phytochemical Screening of *Annona squamosa L*. Fruit Extract

Chemical Constituent	Chemical Test	Observation			Inference
		1	2	3	
Carbohydrates	Benedict's Test	+	+	+	Formation of reddish precipitate
Amino Acids	Millon's Test	+	+	+	Formation of white to red precipitate
Alkaloids	Mayer's Test	–	–	–	No reaction
Flavonoids	Alkaline Reagent Test	+	+	+	Formation of yellow to colourless solution
Phenols	Ferric Chloride Test	–	–	–	No reaction
Tannins	Ferric Chloride-Potassium Ferricyanide Test	+	+	+	Formation of dark blue coloured solution
Terpenoids	Salkowski's Test	+	+	+	Formation of reddish-brown coloured interface

Key: (+) = Present, (–) = Absence

Table 2: FRAP Value of Ascorbic Acid and *Annona squamosa L*. Fruit Extract

Conc. Ascorbic Acid (mg/mL)	FRAP Value (mM Fe (II)/g)	Conc. *Annona squamosa L*. Fruit (mg/mL)	FRAP Value (mM Fe (II)/g)
0.31	3.06 ± 0.005	1.56	0.99 ± 0.004
0.63	4.23 ± 0.006	3.13	1.89 ± 0.004
1.25	5.12 ± 0.004	6.25	3.03 ± 0.004
2.50	6.04 ± 0.003	12.50	5.08 ± 0.005
5.00	7.44 ± 0.007	25.00	6.81 ± 0.005
10.00	9.17 ± 0.010	50.00	8.97 ± 0.005

phenolic compoundspresent in the form of polar glycosides allows the plant to be dissolved easily in a polar solvent such as ethanol (8) andgives antioxidant activity where it has redox potential (12).

Ferric Reducing Antioxidant Power Assay (FRAP)

The FRAP values of ascorbic acid and *Annona squamosa L*. fruit extract are listed in (Table 2). The FRAP values of *Annona squamosa L*. fruit extract show an increase in antioxidant activities in a concentration-dependent manner (Figure 4). The reducing power of *Annona squamosa L*. fruit extract on its ability to transfer electrons to the FRAP reagents increases when the FRAP value increases. The EC_1 value of *Annona squamosa L*. fruit extract and ascorbic acid is 2.131 mg/mL and 0.995 mg/mL, respectively.

Ascorbic acid as the reference standard has a significantly smaller EC_1 value than *Annona squamosa L*. fruit extract. The alkaloid compounds present in *Annona squamosa L*. fruit extract act as a reductantagainst FRAP reagents. It has been suggested that phenolic compounds break the radical chain reaction by the donation of hydrogen atomswhich gives the potential of reducing power (13).

2,2-diphenyl-1-picrylhydrazyl (DPPH)

The percentages for radical scavenging activity (RSA) of ascorbic acid

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and *Annona squamosa L*. fruit extract arerecorded as shown in (Table 3). The radical scavenging activity (RSA) of *Annona squamosa L*. fruit extract shows an increase in direct proportion towards the concentration of *Annona squamosa L*. fruit extract (Figure 5). The lower the absorbance taken during the assay, the higher the percentage of RSA for *Annona squamosa L*. fruit extract.The IC_{50} value of *Annona squamosa L*. fruit extract and ascorbic acid is 4.115 mg/mL and 4.918 mg/mL, respectively. In this study, *Annona squamosa L*. fruit extract has a significantly lower IC_{50} value than ascorbic acid.

Annona squamosa L. fruit extract could scavenge the DPPH radicals' odd electron that shows activities of donation of proton which acts as inhibitors for free radicals. This study suggested that *Annona squamosa L*. fruit extract has a high potency of antioxidant activity in a lower concentration as the IC_{50} value of *Annona squamosa L*. fruit extract is lower than the ascorbic acid as the reference standard(13).

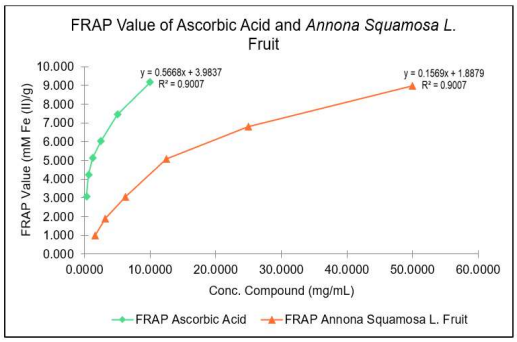

Figure 4: FRAP Value of Ascorbic Acid and *Annona squamosa L*. Fruit Extract, expressed as mM Fe (II)/g of *Annona squamosa L*. Fruit Extract. Values expressed as mean ± SD, n = 3/concentration

Xanthine Oxidase Inhibitory Assay (XOI)

The percentages for the inhibition activity of xanthine oxidase for allopurinol and *Annona squamosa L*. fruit extract are listed in (Table 4). The xanthine oxidase inhibitory (XOI) activity of *Annona squamosa L*. fruit extract demonstrates an increasing concentration-dependent manner of *Annona squamosa L*. fruit extract (Figure 6). The IC_{50} values of *Annona squamosa L*. fruit extract and allopurinol are4.745 mg/mL and 0.154 mg/mL, respectively. The reference standard used is allopurinol have a

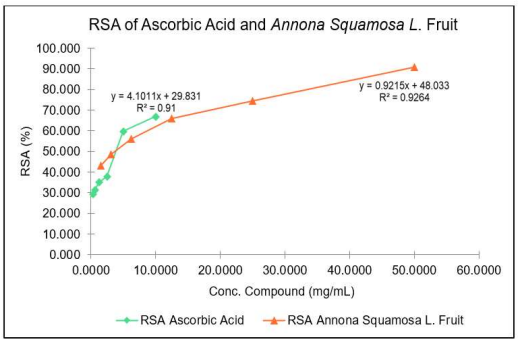

Figure 5: Percentage of Radical Scavenging Activity (RSA) of Ascorbic Acid and *Annona squamosa L*. Fruit Extract. Values expressed as mean ± SD, n = 3/concentration

Table 3: Percentage for RSA of Ascorbic Acid and *Annona squamosa L*. Fruit Extract

Conc. Ascorbic Acid (mg/mL)	RSA (%)	Conc. *Annona squamosa L*. Fruit (mg/mL)	RSA (%)
0.31	29.10 ± 0.004	1.56	42.92 ± 0.005
0.63	31.30 ± 0.005	3.13	48.92 ± 0.004
1.25	34.86 ± 0.004	6.25	56.12 ± 0.003
2.50	37.90 ± 0.003	12.50	65.94 ± 0.004
5.00	59.73 ± 0.004	25.00	74.40 ± 0.004
10.00	66.84 ± 0.007	50.00	90.90 ± 0.005

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Table 4: Percentage for XOI of Allopurinol and *Annona squamosa L*. Fruit Extract			
Conc. Allopurinol (mg/mL)	XOI (%)	Conc. *Annona squamosa L*. Fruit (mg/mL)	XOI (%)
0.03	36.97 ± 0.003	0.31	42.92 ± 0.005
0.06	45.14 ± 0.004	0.63	48.92 ± 0.004
0.13	49.81 ± 0.003	1.25	56.12 ± 0.003
0.25	58.76 ± 0.002	2.50	65.94 ± 0.004
0.50	72.76 ± 0.002	5.00	74.40 ± 0.004
1.00	87.16 ± 0.002	10.00	90.90 ± 0.005

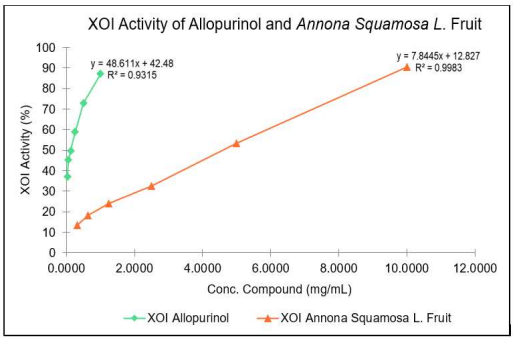

Figure 6: Percentage of Xanthine Oxidase Inhibitory (XOI) Activity of Allopurinol and *Annona squamosa L*. Fruit Extract. Values expressed as mean ± SD, n = 3/concentration

significantly lower IC_{50} value than *Annona squamosa L*. fruit extract.

This study proposed that *Annona squamosa L*. fruit extract has the potential to be a xanthine oxidase inhibitor and an anti-gout agent. A previous study shows*Annona squamosa L*. fruit ethanolic extract has 64.88%xanthine oxidase inhibition power (8). A xanthine oxidase inhibitor possesses the ability to inhibit xanthine oxidase in the pathway of hydroxylation of hypoxanthine to xanthine to uric acid. *Annona squamosa L*. fruit extract inhibits the generation of superoxide anion ($O_{2\text{-}}\bullet$) and hydrogen peroxide (H_2O_2) where both are involved in the catalysation of substrate in the purine metabolic pathway when xanthine dehydrogenase is converted to xanthine oxidase (13).

Conclusion

In this study, *Annona squamosa L*. (sugar apple) fruit has the potential to be a source of antioxidants. The percentage yield of *Annona squamosa L*. fruit extract is 25.58 %. In phytochemical screening,chemical tests for carbohydrates, amino acids, flavonoids, tannins, and terpenoids show positive results. The total phenolic content of *Annona squamosa L*. fruit extract is 8.106 mg GAE/g of *Annona squamosa L*. fruit extract. The EC_1 value of *Annona squamosa L*. fruit extract is 2.131 mg/mL. The IC_{50} values of *Annona squamosa L*. fruit extract in DPPH and XOI assay are 4.115 mg/mL and 4.745 mg/mL, respectively. This study has confirmed the antioxidant properties of *Annona squamosa L*. fruit extract.

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A Qualitative Study on the Implementation of *Sertu* in Pharmaceutical Industry: Processes, Issues and Challenges

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Abstract

Recently, halal pharmaceutical is starting to get the place in the market and is well-accepted among Muslim and non-Muslim consumers. To be certified halal by JAKIM, emphasis is notonly on the origin of the ingredients, but also the manufacturing process whether it complieswith the requirements set by halal pharmaceutical standards. According to Malaysian Standard, MS2424 Halal Pharmaceuticals Guideline, *sertu* cleansing need to be conducted if contamination with severe *najs* (*naj mughallazah*) materials occurs. However, there have been very few studies conducted on halalpharmaceuticals especially on *sertu* cleansing in pharmaceutical industry. Hence, this exploratory qualitative study was conducted to provide insight into the way *sertu* process is carried out in the pharmaceutical industry, and to determine the importantenabling factors needed and the challenges faced in an effort to establish a halal-certified pharmaceutical industry. The data was collected through semi-structured in-depth interviews in two halal-certified pharmaceutical companies in Malaysia. The study used purposive sampling as information could only be obtained from specific target employees inhalal-certified pharmaceutical companies. The results of this study indicate that processes, people, training, records, and materials used for *sertu* are important enablingfactors needed for sertų implementation in pharmaceuticals companies. The study also has identified the effect of *sertu* implementation to Good Manufacturing Practices (GMP) status in pharmaceutical industry and the challenges faced by industry players along the *sertu* implementation process which inludes lack of awareness of *sertu* cleansing by the halal certified pharmaceutical industry players and Muslim consumers, issues with the standard and financial issues in implementing the *sertu*. A proposed conceptual framework was developed from the findings of the study.

Keywords: Halal pharmaceuticals, Halal medicines, Seru cleansing, Halal pharmaceutical industry, Good Manufacturing Practice(GMP)

Introduction

Malaysia has become a leading country among several other countries (e.g. Egypt, Singapore, Indonesia etc.) in incorporating halal value in pharmaceuticals and cosmetics productions (1).Halal pharmaceuticals provide a way for Muslim consumers to preserve their faith and belief even in health care practices (2) It should be free from haram constituents and tayyib, which means they have to be clean, pure and produced based on standard processes and procedures (3). Halal pharmaceuticals are produced based on the harmonization of Islamic religious law, GMP standards as well as the approved halal supplier and material list (4).

"Halal" is an Arabic term meaning permitted, and the opposite is "haram" meaning unlawful for a Muslim based on the Quran (5).The MS2424 requires a product to be approved by National Pharmaceutical

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Regulatory Agency which signify safety, quality, and efficacy and comply with the Pharmaceutical Inspection Convention and Pharmaceutical Inspection Co-operation Scheme (PIC/S) standards first before it can be certified halal (6). The MS2424 Halal Pharmaceuticals General Guidelines defines halal pharmaceutical as a product that containing ingredients permitted by Islamic law and fulfilling the following conditions(7).

a) Any parts or products of animals used should be halal and slaughtered according to Shariah Law;
b) Free from *najs* according to Shariah Law;
c) Possess no harm to human health in accordance to prescribed dosage;
d) Are prepared, processed and manufactured using equipment that are free from *najs* according to Shariah Law;
e) Free from human parts or its derivatives that are not permitted by Shariah law; and
f) Halal pharmaceutical products should be physically separated during its preparation, processing, handling, packaging, storage and distribution from any other pharmaceutical products that do not fulfil the conditions stated in items a), b), c), d) or e) or any other items that have been decreed as non-halal and *najs* by Shariah law.

A halal product will lose its purity if contamination with non-halal substances occurs.The storage, equipment and handling facilities which have been contaminated with severe *najs* (contaminant of porcine and canine origin) need to be cleansed by *sertu* cleansing for purification using water mixed with clay(8). However, in pharmaceutical productions, the possibility of the products to be contaminated by the *sertu* clay need to be considered so that high-quality and safe pharmaceutical products can be produced. In addition, pharmaceutical productions also often involve with the use of sensitive equipment that require extra precautions during its cleaning and handling. The use of clay in *sertu* cleansing could cause scratch and damage to the equipment if the cleaning process is not conducted in an appropriate manner. Therefore, a standard operating procedure (SOP) for *sertu* that would comply with both GMP, and halal requirements should be developed by the organizations involved in order to implement *sertu* guidelines effectively.

Sertu (ritual cleansing), is a Malay term, defines as Islamic method of cleansing to eliminate*mughallazahnajs* (e.g. dogs, pigs, or their descendants) by washing contaminated surfaces with water mixed with soil (one time) followed by clean water (six times) (9, 10).*Sertu* cleansing is crucial to ensure toharah (purification) in Islam so that ibadah (worship) will be accepted (8).

According to MS2424 Halal Pharmaceuticals Guidelines, the general requirements of *sertu* cleansing include (7):

a) Seven times washing, one of which shall be water mixed with soil,
b) The first wash shall be to clear existence of *najs*, even if a few washes are needed. The water from the first cleaning shall not remain behind and the next wash shall be counted as the second wash.
c) The amount of soil used is just enough to sufficiently change the physical appearance of water from clear to turbid.
d) The usage of cleansing agent containing soil is permitted.

Sertu cleansing will not only remove the existence of *najs* from the view of religious faith, but also the negative energy from it. Contamination by *najs* may occur at any stage along the halal supply chain processeswhich involve the use of transport and containers, warehouse, surrounding areas and infrastructure (e.g. floors, loading bays, storage rooms) (11).

Traditionally, any fixed concentration of clay can be used for *sertu* cleansing. However, some modifications need to be conducted for industrial applications sinceimproper selection of clay can cause physical damages (e.g. rust, scratch, and blockage) on sensitive and expensive instruments as well as harmful to human (9). Corrosion can occur at the minimum level in

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the pH range of 6-12 of the clay, while rust can rapidly occur outside this range which would be fasten by very acidic or alkaline conditions(12). Smaller clay particle size will increase the clay's ability to absorb and remove impurities due to its large surface area. Clay also can regulate the flow of cleaning products on the target surfaces due to its impacts on the viscosity (9). Heavy metals contaminants of the clay (i.e. mercury, lead) must not exceed the allowable limit as they can accumulate in the body if they are ingested. The clay also shouldhave a low moisture content so that it could stand for a longer-term storage condition (9). Surface properties such as the chemical composition, phase composition, and roughness can affect cleansing ability of the soil on the glazed surface. Repeated soiling and cleaning showed accumulation of soil on surfaces with highest roughness (13).

Issues and Challenges in *Sertu* Implementation

Some pharmaceutical companies need to spend up to thousands of U.S. dollars per year to meet halal requirements stated in the guideline including replacement of any prohibited ingredient which has been identified during ingredient's information review and the facility audit (14). In addition, Halalstandards also need to be implemented parallel with other recognised international standards such as Hazard Analysis Critical Control Point (HACCP) and Good Manufacturing Practice (GMP) to consistently produce medicinal products to the quality standards appropriate to their intended use and as required by the marketing authorisation or product specification (7, 15, 16). Lack of understanding in halal (permissible) and its association with tayyiban (wholesomeness), especially among the manufacturers, service providers and consumers could present threats to the success of halal productions. Lack of understanding of the halal procedures could contribute to a slow process and minimizes the number of halal certificate approval. (17, 18) Similar understanding and operational practice of handling halal products between all parties are important to maintain an intact halal integrity from upstream to downstream supply chain.(19). Hence, this study is conducted to understand the processes, issues, and challenges in implementing *sertu*according to the guidelines in the MS2424 Halal Pharmaceuticals Standard in halal-certified pharmaceuticals companies in Malaysia.

Materials and Methods

This was qualitative research since there was very few research done on the *sertu* cleansing. It is exploratory in nature and is very useful since not much is known about targeted phenomenon until now (20). The study used purposive sampling and took place in two pharmaceutical companies located in Selangor which are Company A and Company B withthe targeted employees who are knowledgeable about the *sertu* process to obtain a maximum insight for the case studied(21). Table 1 summarizes the participants' information.

Semi-structured in-depth interviews were conducted to obtain a rich and in-depth information about the experiences of the individuals. It can occur either with an individual or in group (22). A total of three individual interview sessions were conducted with the employees from Company A and a focus group interview was conducted with the employees from Company B. The interviews were guided by semi-structured interview protocol previously constructed based on literature reviews. Each interview session lasted between thirty minutes to one and half hour, at a time, by using combination of Malay and English language. Notes were taken during the interviews, and the interviews were recorded with the participants' permissions for later transcription (23). An extensive review on relevant literatures and previous studies retrieved from different databases were performed before and during data collectionto gain better understanding on the subject matter (24).

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Table 1: The participants' information for Company A and Company B

Company	Type of company	Position	Years in Industry
Company A	Halal-certified Pharmaceutical Company	Microbiologist in QC	3 years
Company A	Halal-certified Pharmaceutical Company	Halal Executive	8 years
Company A	Halal-certified Pharmaceutical Company	QC Technician	6 years
Company B	Halal-certified Pharmaceutical Company	Top Management Level	21 years
Company B	Halal-certified Pharmaceutical Company	Secretary for Halal Council	9 years (in halal)
Company B	Halal-certified Pharmaceutical Company	Senior Manager/ Shariah Advisor for Halal Council	12 years

Results and Discussion

In the study, the data was analysed inductively started with gathering all information related to the case including the transcripts, records, the investigator's own documents, and voice recorded interviews. The data was managed manually with the help of Microsoft Word rather than with the use of computer programmes (eg.ATLAS.ti, Nvivo) to eliminate the gap between the researcher and the data (25). Thematic analysis was conducted by using coding method (open coding and axial coding) to categorize the data that seems related to each other into categories/ theme (26).

Enabling Factor of *Sertu*

Five requirements as the enabling factors were formulated from the interview sessions: the processes, the people, the training, the records, and the materials used for *sertu*.

Processes

Sertu cleansing in Company A was conducted due to the history of using non-halal materials (e.g. raw materials, media) in production. It was carried out by 15 employees in the microbiology lab and 30-50 employees in the production line. The whole process was observed by Selangor Islamic Affairs Department (JAIS) staff and halal executive officer. It began with the investigation of the processes and machines that had been contaminated with non-halal materials and removal of all non-halal materials. The operations in the affected areas were stopped for a week to allow the *sertu* cleansing (two days) and environmental monitoring (five days) to be conducted (Figure 1).

First, washing of equipment was conducted with clay water which had been prepared earlier by JAIS followed by another six times of washing with clean running water (by spraying with clean water for sensitive equipment). The water was allowed to spread evenly over the surface of the equipment.The equipment was wiped immediately after each spray by using clean cloth to prevent any damage occurring. All lab coats were soaked in the clay water (first washing) followed by in the clean water (subsequent six washing). The tables were wiped by using clean clothes which had been soaked in the clay water (first washing) followed by another six times washing with clean water by using the new clean cloth. Cleaning as per Standard Operating Procedure (SOP) and environmental monitoring were conducted after the *sertu* cleansing has completed (after drying and assembling).

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Sertu cleansing in Company B was conducted for peace of mind since the company has been involved in manufacturing for quite a while where there is a possibility that contamination may have occurred during that time. The production process in Company B was stopped for a week to allow sertu cleansing (two days) and environmental monitoring (five days) to be conducted. The number of staff involveddepends on the number of equipment and the size of the affected areas. The clay water was prepared by halal executive and verified by shariah advisor of the company under JAIS's supervision. Before it was used for *sertu* process, the clay undergone sterilization by autoclaving. Microbial tests were conducted on the clay before and after sterilization to ensure the safe use of the clay. The employees learned on-the-spot on how to perform *sertu* by using three cleansing methods (washing, spraying and rinsing) on the actual day of the *sertu* process. The whole cleansing process was observed by officers from JAIS. The GMP cleaning and environmental monitoring were conducted after the cleansing process was completed. GMP cleaning also was conducted before the *sertu* cleansing. The company need to pass the environmental monitoring (e.g. swab test, settle plate) before the operation can be started.

People

Sertu process in Company A was led by the Head of Production and halal committee members and supported by JAIS. A briefingwas given by JAIS before the*sertu* process was conducted. The employees were divided earlier into groups and each group was guided by staff from JAIS. The company also was guided and advised by Halal Industry Development Corporation (HDC) during the implementation process. The shariah competent authorities (JAKIM, Selangor Islamic Religious Council (MAIS)

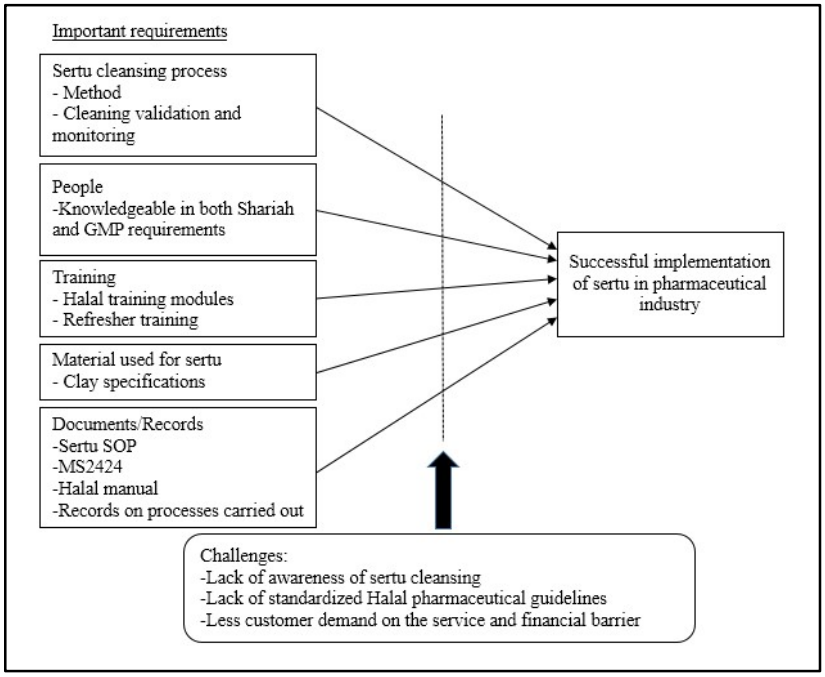

Figure 1: Proposed conceptual framework for important requirements and challenges in *sertu* implementation in pharmaceutical industry

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and JAIS), members of Halal Council and site Halal Committees, Group Halal Manager, production members, Quality Assurance (QA) members and Quality Control (QC) members were involved in the *sertu* implementation of Company B. Exchange of knowledge between the employees and staff from JAIS occur along the process (Figure 1).

Training

One of the participants from Company A mentioned that all employees including cleaners and non-Muslim employees were provided with two hours training (including evaluation) on introduction of halal and *sertu* SOP, in addition to the yearly refresher training. Other than that, an induction program also is provided for new employees. An external trainer from HDC also was invited to provide halal training modules for the employees in the company. On the other hand, the participant from Company B stated that practical guidance and training were provided by JAIS for members of Halal Council and site Halal Committees, Group Halal Manager, production members, QA and QC. The training was conducted not later than the day before *sertu* was conducted. Refresher trainingalso was provided for the staff. According to Ahmad&Mohd, 2016, suggested that training could contribute to a successful halal ritual cleansing implementation by enhancing halal integrity through creating awareness among employees (Figure 1).

Records

Standard Operating Procedure (SOP) for cleaning and for *sertu*, Halal Assurance System (HAS) and other documents such as halal manual, MS2424, and work instruction were used to guide the *sertu* process in Company A. SOP on how to purchase items such as raw materials and packaging materials was referred to guide the purchasing process. A plan with proper timeline that includes the date for conducting actual *sertu* process and when it has been planned was prepared earlier by the employee. In Company B, checklists and reports were prepared to ensure the *sertu* process adhered to GMP requirements and a proper documentation for shariah requirements has been done for future references. Any documents on *sertu* were prepared by halal executive and verified by shariah advisor and technical advisor, whereas any documents related to GMP were prepared by QC personnel and verified by QA head (Figure 1).

Materials used for Sertu

Fine clay powder together with the Certificate of Analysis (COA) and Material Safety Data Sheets (MSDS) that *provide information about specifications and safety status of the clay* were provided by JAIS before *sertu* was conducted in Company A. According to people in charge, 2015, any processing aids, hazardous materials, other special materials, or materials transferred to another unit within the company's control are not required to undergo testing if the manufacturer's certificate of analysis is obtained. In Company B, the water and clean clay powder that were used for the *sertu* process had been earlier approved by the competent authorities which are JAKIM, MAIS, and JAIS. The clay undergone microbial test before and after sterilization to ensure the safe use of the clay. Sterilization by autoclave was conducted on the clay before it was used for the *sertu* process. The clay should pass the tests required by GMP guidelines before it can be used in the *sertu* process. It should meet the required criteria in terms of low heavy metal contaminants, slightly acidic to neutral pH, fine particle size and low moisture content so that it would fulfill the standard halal requirements on quality and safety of the products (9).

Impact of *Sertu* Implementation to the Good Manufacturing Practice (GMP) Status in the Selected Pharmaceutical Companies

In order to comply with all GMP requirements, Company B ensured that the equipment, utilities, utensils and clothes used in the *sertu* process follow the

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pharmaceutical standard. Cleansing of equipment was conducted in washing areas that comply with GMP standard to prevent any cross contamination occurs. Appropriate gown was worn during the *sertu* process. GMP cleaning and environmental monitoring was conducted after *sertu* cleansing has been completed. Microbial test was conducted on the clay before and after sterilization as required by GMP guideline. The participants from Company A said there was no issue on *sertu* cleansing raised by theregulatory authorities and health authorities (e.g. Kementerian Kesihatan Malaysia (KKM), GMP, Scientific and Industrial Research Institute of Malaysia (SIRIM), National Pharmaceutical Regulatory Agency (NPRA), ISO 9001, ISO 70025, ISO 4001, ISO 14001, and ISO 17025). There was also no issue raised by the auditors from other countries as well such as Pfizer. Other than that, the process didnot cause any damageand scratch to the equipment because fine particles of clay were used for the *sertu* process.

Specific Issue/s and Challenge/s in Implementation of *Sertu* Procedures in Selected Halal Pharmaceutical Companies

The issues faced by the participants are listed, compared and any repeated issues are removed from the lists. Table 2 shows the listing of issues faced in *sertu* cleansing in halal pharmaceutical industry. All issues are coded Issue # and classified into categories coded as Barrier #.

All the above issues are then grouped into three larger groups of issues.

Table 2: Issues faced in sertu implementation in halal pharmaceutical industry

Codes	Issues Identified
Issue 1	Lack of knowledgeable person in charge of halal related matters (eg.*Sertu* cleansing) at the beginning
Issue 2	Lack of information available on *sertu* implementation in pharmaceutical settings
Issue 3	No experience performing *sertu* cleansing in pharmaceutical settings
Issue 4	No example of *sertu* cleansing in halal certified pharmaceutical company as a benchmark
Issue 5	Lack of industrial awareness of the halal concept throughout the whole supply chain
Issue 6	*Sertu* is an expensive method; not cost-effective (eg. Off operation for a week, discard non-halal materials, change of gowning)
Issue 7	Increase in workload before (eg. Set up tent, arrangement of equipment) and after *sertu* (eg. GMP cleaning)
Issue 8	Time-consuming (eg. New media validation, cleaning of small apparatus)
Issue 9	Existing references (eg MS2424, *sertu* SOP) are too general
Issue 10	Lack of standardized halal pharmaceutical guidelines
Issue 11	Lack of samak clay standard
Issue 12	Difficulty to look for the right *sertu* material that comply with GMP (sterilized) and Shariah (‘an acceptance) requirements
Issue 13	Problems in finding competitively priced halal-certified suppliers
Issue 14	Problem with current suppliers (eg non-muslim suppliers)
Issue 15	Lack of economy of skill since halal pharmaceutical is still in the infancy
Issue 16	Halal is currently not considered as a preferential criterion in the purchasing of pharmaceutical products

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Issues 1, 2, 3, 4 and 5 are grouped in Barrier 1 which indicates the lack of awareness of *sertu* cleansing by the halal certified pharmaceutical industry players and Muslim consumers. Issues 9, 10 and 11 are grouped in Barrier 2 which specifies the issues with the standard. Issues 6, 13, 14 and 15 are grouped in Barrier 3 which concerned with financial issues in *sertu* implementation in halal pharmaceutical industry. The result is as shown in Table 3 (27).

Barrier 1 discusses the awareness among halal pharmaceutical industry players and Muslim consumers. Studies fromMahidinet al (28) and Wahab (29) described lack of knowledge, awareness and understanding of the halal concept among Muslimconsumers and the manufacturers may become the reason for the loss of appreciation to halal. As mentioned by participants from Company B, halal is currently not a preferential criterion in the purchasing of pharmaceutical products. Lack of industrial awareness is not limited to food and beverages, or porcine and alcohol-based materials, but throughout the whole supply chain. Other than that, the participants from Company A and B mentioned that there isalso a lack of relevant information on *sertu*cleansing for pharmaceutical settings that could be obtained from other sources (e.g. internet). The participants from Company A described the difficulty of implementing*sertu* in the organization at beginning due to the absence of knowledgeable person in halal related matters among them. They also were unable to set other companies as a role model due to the confidentiality of the information needed from them.

Barrier 2 explains the issues related to the standard on halal pharmaceuticals. The participants from Company A and B commented on MS2424 which are too general and not standardized between countries. They were not convinced with the products from other countries in the market and there were also some complaints received from other countries due to the different guidelines used between countries as well. As proposed byNasaruddin et al (30) there are still insufficient development of

Table 3: Categories of issues in sertu implementation in halal pharmaceutical industry

Lack of awareness of sertu cleansing by the halal certified pharmaceutical industry players and Muslim consumers (Barrier 1) ▪ Lack of knowledgeable person in charge of halal related matters (eg.Sertu cleansing) at the beginning (Issue 1) ▪ Lack of information available on sertu implementation in pharmaceutical settings (Issue 2) ▪ No experience performingsertu cleansing in pharmaceutical settings (Issue 3) ▪ No example ofsertu cleansing in halal certified pharmaceutical company as a benchmark (Issue 4) ▪ Lack of industrial awareness of the halal concept throughout the whole supply chain (Issue 5)
Issues with the standard (Barrier 2) ▪ Existing references (eg MS2424, sertu SOP) are too general (Issue 9) ▪ Lack of standardized halal pharmaceutical guidelines (Issue 10) ▪ Lack of samakclay standard (Issue 11)
Financial issues (Barrier 3) ▪ Sertu is an expensive method; not cost-effective (Issue 6) ▪ Problems in finding halal-certified suppliers (Issue 13) ▪ Problem with current suppliers (eg non-muslim suppliers) (Issue 14) ▪ Lack of economy of skill since halal pharmaceutical is still in the infancy (Issue 15)

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halal procedures as well as guidelines from the ground up and current guidelines are still lacking halal concept. Consequently, Muslims have put less attention on halal production, and they are still facing halal problems. Lack of standardized halal guidelines also could lead to prolonged process and higher costs of production(21). Other than that, the participants from Company A also mentioned the lack of samakclay standard that can be used for validation purposes. A specific samak clay standard for industrial application is needed to meet the halal requirement and alsothe specifications of the equipment or machines(27).

Barrier 3 discusses the financial issues encountered to implement *sertu* in pharmaceutical industry. Participants from Company B commented on*sertu* as an expensive method because they have to stop production for a week. In addition, they also expressed the difficulty in findinga competitively priced halal certified ingredient supplier. The statement was supported by participants from Company A which found that looking for a halal certified supplier was time-consuming.Other than that, they also need to remove all non-halal materials, and make changes of gowning before they come up with the decision to establish a halal-certified company. The scenario stated above is supported by Hanzaee&Ramezami (14) which mentioned about some pharmaceutical companies that need to invest more in order to replace the ingredients that do not meet the criteria underlined by the guidelines which have been identified during ingredient's information review and the facility audit.

Conclusion

In order to meet consumers' demand on halal pharmaceuticals, pharmaceutical companies need to strictly follow the requirements highlighted by halal pharmaceutical standards MS2424. This include the need to ritually clean the equipment, machine appliances and processing aids which have been contaminated with non-halal substances. For the first research question, five requirements that contribute to the successfulness implementation of *sertu* in halal pharmaceutical industry were identified. The five requirements including process, people, training, records, and material for cleansing. A conceptual diagram was developed from the findings. For the second research question, the effect of *sertu* cleansing on GMP was examined. It is clear that *sertu* cleansing does not affect the GMP status nor cause any damage to the sensitive machines due to the use of clay in the cleaning process and is safe for the pharmaceutical products. For the third research question, there are three issues have been identified in *sertu* cleansing implementation in halal pharmaceutical industry. These issues are lack of awareness of *sertu* cleansing by the halal pharmaceutical industry players and Muslim consumers (Barrier 1), issues with the information in standards (Barrier 2), and financial issues (Barrier 3). These issues need to be addressed and further studied in order to implement *sertu* successfully in halal pharmaceutical industry.

This study is expected to provide insight on *sertu* cleansing in halal pharmaceutical industry that can be referred by future researchers and other pharmaceutical manufacturers that might be interested in halal pharmaceuticals production. This indirectly helps in the development of halal pharmaceuticals industry in Malaysia.

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Formulation and Evaluation of Halal Hair Growth Promoting Shampoo Containing *Centella asiatica* and *Phyllanthus emblica*

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Abstract

Halal awareness among Muslim consumers has widened to a wide range of products including personal care and cosmetics. Shampoo is the most frequently prescribed treatment for hair and scalp conditionswhile *Centella asiatica* and *Phyllanthus emblica* have been found to have antioxidant properties thatexhibit positive benefits on hair. The aim of this study is to develop halal hair growth promoting shampoo containing *P. emblica* and *C. asiatica*. Halal certificate, Certificate of Analysis and Material Safety Data Sheet were requested from the suppliers for halal evaluation of the ingredients. Seven shampoo formulations (F1-F7) were created with varying concentrations of *P. emblica* and *C. asiatica* extracts, while one formulation was left as a blank. Then, the shampoo formulations underwent an organoleptic evaluation along with other tests for dirt dispersion, pH, solid content, foaming capacity, and stability. The 2,2-diphenyl-1-picrylhydrazyl (DPPH) method was used to measure the antioxidant activity. All of the ingredients used to formulate the shampoo are considered halal based on certificate, origin, and their composition. The evaluation of the shampoo demonstrated that all the formulations exhibited ideal physicochemical properties for hair cleansing and F3 with 4% of *C. asiatica* extracts and 2% of *P. emblica* extract produced the best antioxidant activity as it inhibited 92.5% of DPPH. Overall, this study provides the requirement on formulation of halal hair growth promoting shampoo containing *P. emblica* and *C. asiatica*. The results show that the formulated shampoo is halal based on document review, exhibited high antioxidant properties for hair growth promoting effect and possess good characteristics as cleansing agent.

Keywords: Halal, shampoo, antioxidants, hair growth, *Centella asiatica, Phyllanthus emblic*

Introduction

Halal is an Arabic word that refers toany object or an action which is permissible to use or engage in, according to Islamic law, whereas haram is anything unlawful or forbidden(1). Muslim consumer halal awareness has widened from being concerned with meat-based products to a wide range of products today where recent research has cited that more than 20% of Muslim consumers are concerned about halal issues with the products they are using.

Cosmetic products refer to any substance or preparation intended for application to any external part of the human body or the teeth or buccal mucosa mainly for the purpose of cleaning, promoting attractiveness, perfuming, or protecting and keeping them in good condition (2).The halal aspects of cosmetics cover ingredients, safety and all the processes involved in production right up to delivery to consumers.

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A basic concept shared by most halal certifiers of personal care products is vigilance regarding ingredients and their origins, as well as the manufacturing process, to check for potential points of contact with haram products (3).

Shampoo represents the largest segment of hair cosmetics (4) and the treatments are the most commonly used means of managing hair and scalp conditions(5). Shampoo helps to remove dirt, oils, dandruff, skin particles, and other contaminants that gradually build up in hair as well as leaving the hair in a satisfactory condition after rinsing so that it can be combed easily both in the wet and dry state (4).

Hair is derived from the ectoderm of the skin and hair loss affects millions worldwide due to aging, hormonal dysfunction, medications, and supplements, or as a side effect of cancer treatment (6). Molecules that can promote hair follicle stem cell activation have been intensely searched for, as they may help provide therapeutic and cosmetic interventions(6). The drugs of synthetic origin that are approved by the FDA for hair growth are associated with potential side effects (7). Thus, people are interested in the usage of alternative remedies which are herbal hair growth formulations(8).

Medicinal herbs producing good antioxidant activities have been employed as the source of natural antioxidants (9). Studies show that there is a strong connection between antioxidant activity and hair growth promoting effects. The best ingredients are antioxidants that can interrupt radical chain processes, fight free radicals in our body that cause the hair follicle cells in the scalp to break down, help to repair hair systems, protect against oxidative damage as well as increase the blood circulation and thus help in hair growth(10).

Centella asiatica is a medicinal plant that has been used for hundreds of years due to its health-promoting effects, especially in dermatological conditions. It can prevent hair loss as polyphenol, flavonoid, and vitamin C compounds are abundant in *C. asiatica*, contributing to its significantly higher antioxidant activity (11).

Phyllanthus emblica highly contains vitamin C and low molecular weight hydrolysable tannins making it a good source of antioxidant (12). Other than helps to maintain vernal hair color and retards premature graying, it also supports the strength of the hair follicles, so there is less thinning of hair with age(13).Although there are many treatments available, many people are still suffering from hair loss. Therefore, it is important to develop novel shampoo formulations that prevent hair loss and promote hair growth (8). At the same time, Muslims would want to be certain that the cosmetic and personal care products they use are halal (1). *P.emblica*has shown prominent antioxidant effects which can promote hair growth and prevent graying of the hair. *C. asiatica* contains essential oils, sterols, flavonoids, glycosides, and triterpenoid saponins and is commonly used in hair care formulations. It is high in antioxidants hence helps to prevent greying of hairs as well as promote hair growth. However, the combination of *P. emblica*and *C. asiatica* in shampoo formulation is not yet available in the market.

Materials and Methods

Materials

C. asiatica extract was bought from Xi'an SR Bio Engineering in China, while *P. emblica* fruit powder was bought from Bio Organic in Sri Petaling, Kuala Lumpur.Sodium lauryl ether sulphate, polysorbate-20, DMDM hydantoin and EDTA were obtained from Ken Prima (Malaysia). Cocamidopropyl betaine and sodium chloride were purchased from Personal Formula Resources (Malaysia). 2,2-Diphenyl-1-picrylhydrazyl (DPPH), ethanol and ascorbic acid were purchased from R&M Chemicals (Malaysia).

Methods

Identification of halal ingredients

Three documents that were requested together with each ingredient are Halal Certificate, Certificate of Analysis and

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Material Safety Data Sheet. Hence, the ingredients that are halal certified by JAKIM or recognized by JAKIM were preferred.

Preparation of shampoo

Preparation of *P. emblica* extract

The extract of *P.emblica* was prepared using a decoction method. As shown in (Table 1), specific amounts of P. emblica powder and water were used for each shampoo formulation. Following a procedure adapted from previous research (14) with minor modifications, the powder was first dissolved in water in a beaker. The mixture was then heated to 65 °C for 15 minutes and allowed to cool. After cooling, the herbs were filtered out to obtain the extract. This extract was then concentrated to one-fourth of its original volume. The final prepared extract was subsequently added to the base shampoo formulation.

Table 1: Preparation of *P. emblica* extract

Formulation	*P. emblica* powder (g)	Distilled water (ml)
F1	-	-
F2	1	10
F3	2	20
F4	3	30
F5	4	40
F6	5	50
F7	6	60

Preparation of blank shampoo and incorporation of plant extracts

The formulation of the blank shampoo was adapted from previous research (15) with some modifications. Seven shampoo formulations (F1-F7) were created with varying concentrations of *P. emblica* and *C. asiatica* extracts, while one formulation was left as a blank. Initially, SLES, cocamidopropyl betaine, and polysorbate-20 were combined and dissolved in water with continuous stirring. Next, DMDM hydantoin and EDTA were added and stirred until a uniform solution was achieved. The powdered extracts were then dissolved in water and incorporated into the formulation (Table 2). The pH of the mixture was

Table 2: The composition of ingredients used in the formulation

Ingredients	Function	Blank	F1	F2	F3	F4	F5	F6	F7
Distilled water	Solvent	q.s. 100	q.s. 100	q.s. 100	q.s. 100	q.s. 100	q.s. 100	q.s. 100	q.s. 100
SLES	Primary surfactant	20	20	20	20	20	20	20	20
Cocamidopropyl betaine	Secondary surfactant	10	10	10	10	10	10	10	10
Polysorbate-20	Solubilizer	6	6	6	6	6	6	6	6
DMDM hydantoin	Preservative	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
EDTA	Chelating agent	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Sodium chloride	Thickener	1.8	1.8	1.8	1.8	1.8	1.8	1.8	1.8
C. asiatica	Active ingredient	-	6	5	4	3	2	1	0
P. emblica		-	0	1	2	3	4	5	6

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adjusted using TEA or citric acid, and finally, sodium chloride was added to adjust the viscosity.

Evaluation based on physicochemical properties

Organoleptic properties

The formulations will be evaluated in terms of their clarity, color, odor, homogeneity, viscosity, and consistency(16).

pH test

The pH meter is calibrated using standard buffer solution, pH of 10% v/v shampoo solution in distilled water was measured at room temperature. The pH of tested commercial shampoos was found within the preferred range between 5.5 to 6.5.(1).

Foaming ability and stability

Foaming ability was determined by using the cylinder shake method. Briefly, 50 mL of the 1% of formulated shampoo solution was placed into a 250 mL graduated cylinder; it was covered with one hand and shaken 10 times. The total volume of the foam content after 1 min of shaking was recorded. Foam stability was evaluated by recording the foam volume after 1 minute and 4 minutes of shake test(17).

Dirt dispersion

Two drops of shampoo were added in a large test tube containing 10 ml of distilled water. I drop of India ink was added, the test tube was stoppered and shake it ten times. The amount of ink in the foam was estimated as None, Light, Moderate, or Heavy (18, 19).

Solid content

A dry porcelain dish was taken, and 4 grams of each shampoo formulation is poured in it. The exact weight of the porcelain dish was noted. The dish is then put for evaporation in the oven at a temperature of 105°C for 3 hours till the whole liquid has evaporated. The amount of solid left drying was determined (18, 20).

Antioxidant study

Antioxidant activity of the shampoo formulation is examined on the basis of scavenging effect and hydrogen donating ability on the stable of 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical. Ethanol is used as blank sample and ascorbic acid as positive control. The antioxidant activity is determined by using the equation to determine the reduction in absorbance at various concentrations. The method of sample and ascorbic acid preparation are adapted from Joshi *et al*. (2018)(17).

Preparation of sample

A 10 mg/ml shampoo formulation was prepared using ethanol as a solvent, with the volume adjusted to 10 ml in a volumetric flask. A 0.004% DPPH solution was made by dissolving 0.4 mg of DPPH in 100 ml of 70% ethanol. To each sample, 1.5 ml of the prepared DPPH solution was added to 0.5 ml of the shampoo formulation and vortexed. The mixed solution was then placed in a dark room at room temperature for 20 minutes. After this period, UV spectroscopy at 520 nm was used to measure the absorbance of each solution. The free radical scavenging activity of DPPH was calculated using the formula:

$$\% \text{ inhibition} = \frac{\text{A DPPH} - \text{A Sample}}{\text{A DPPH}} \times 100$$

Where,
A DPPH = Absorbance of DPPH
A Sample = Absorbance of sample
Each measurement was repeated three times.

Preparation of Ascorbic acid

A 10 mg/ml solution of ascorbic acid was prepared in ethanol. From this solution, 5 ml were taken and diluted to a final volume of 50 ml. Various concentrations were then prepared from this stock solution, specifically 20 µg/ml, 40 µg/ml, 60 µg/ml, 80 µg/ml, and 100 µg/ml.

Results and Discussion

Evaluation of Halal Status of Shampoo Ingredients

Three documents that were requested upon purchasing the ingredients

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are Halal certificate, Certificate of Analysis (CoA), and Material Safety Data Sheet (MSDS). The list of documents that are available and provided by the suppliers is shown in (Table 3).

The organizations recognized by JAKIM are listed in The Recognized Foreign Halal Certification Bodies and Authorities as of December 1st, 2020. SLES, polysorbate 20, DMDM hydantoin, EDTA, and sodium chloride are considered halal ingredients as they are halal-certified by the organizations that are recognized by JAKIM. According to the documents, the composition of this ingredient is 35% CAPB and 65% water and does not contain animal-derived products. Thus, this ingredient is considered halal. *C. asiatica* extract is halal certified by IFRC Hong Kong. This organization strictly follows the Malaysian Standard and World Halal Council Standard as well as received recognition by Islamic Religious of Singapore (MUIS) and Korea Ministry of Food & Drug Safety (KMFDS). The *P. emblica* extract is handpicked, 100% certified organic by ECOCERT, USDA, India Organic, and FSSAI, purely natural and safe for consumption. According to the website, this extract is also cruelty-free and vegan. It has not been tested on animals and contains no animal parts or extracts.

This study lack of information regarding the manufacturing process of each ingredient. Knowing the origin of raw materials and the production process of cosmetic ingredients is vital for Muslim consumers(21). As a result, some excipients were purchased despite the fact that they did not have a halal certificate and no information on the manufacturing process. Thus, CoA and MSDS were required to ensure that the ingredients' origin, safety, and composition meets the product quality standards.

Evaluation of shampoo

Organoleptic properties

The formulated shampoos were evaluated for physical characteristics such as color, odor, homogeneity, viscosity, and consistency (Table 4). Figure 1 shows organoleptic properties of the shampoo formulations.

Based on Figure 1, the addition of extracts turned the clear shampoo into brown due to the colour of the extracts. F1 (6% of

Table 3: List of documents received from the supplier for halal evaluation

Ingredients	Halal Certificate		CoA	MSDS
	Availability	Organization		
SLES	/	The Central Islamic Council of Thailand*	X	X
Polysorbate-20	/	Islamic Religious Council of Singapore*	X	X
Cocamidopropyl betaine	X	-	/	/
DMDM hydantoin	/	The Indonesian Council of Ulama*	X	X
EDTA	/	Halal Quality Control*	X	X
Sodium chloride	/	ARA Halal Certification Services Centre Inc.*	/	/
C. asiatica	/	Islamic Food Research Centre, Hong Kong	/	/
P. emblica	X	-	X	X

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Table 4: Organoleptic properties of formulated shampoo					
Formulation	Colour	Odour	Homogeneity	Viscosity	Consistency
Blank	Clear	Good	Good	Good	Liquid
F1	Dark brown	Good	Good	Good	Liquid
F2	Dark brown	Good	Good	Good	Liquid
F3	Dark brown	Good	Good	Good	Liquid
F4	Dark brown	Good	Good	Good	Liquid
F5	Dark brown	Good	Good	Good	Liquid
F6	Dark brown	Good	Good	Good	Liquid
F7	Light brown	Good	Good	Good	Liquid

Figure 1: Physical appearance of formulated shampoo

CAE) has the darkest colour while F7 (6% of PEE) has the brightest colour among all formulations. However, there were no trend in changes of colour between F2 to F6 which might be due to a little difference in extract content across the shampoo formulations. No significant difference was observed in terms of odor, homogeneity, viscosity, and consistency between all the formulated shampoo except for color and all the formulations show good characteristics. These parameters were evaluated by vision and touch sensation.

pH

The pH level of all formulated shampoo containing different concentrations of *C. asiatica* and *P. emblica* are tabulated in (Table 5). The pH range of hair is 4.5-5.5 and the optimum pH for shampoo is 5.5 to 6.5(1).

Based on the results, pH levels of all formulated shampoo containing different combinations of *P. emblica*and *C. asiatica* extract are acidic compared to the blank shampoo which is more alkaline. The pH level of F1, F2, and F7 are within the desired range, while pH of F3, F4, F5, and F6 are slightly acidic and below the optimum range. The pH level of all the tested shampoos is slightly acidic compared to the blank shampoo formulation due to the acidic nature of the extracts. F1 and F7 are single formulation of shampoo containing *C. asiatica* and *P. emblica* extracts respectively. It shows that combination of the extracts produced more acidic shampoo instead of its single formulations. One method for reducing hair damage is to adjust the pH. The pH of the acidic shampoo can be adjusted to the optimum level by the addition of triethanolamine solution to prevent any irritation and damage due to the consumption of the shampoo that is too acidic (22).

Foaming ability and stability

Results of foaming ability and stability test for all formulated shampoos are shown in (Figure 2). From the results, generally, the volume of foam produced by F1, F4, F5, F6, and F7 remain constant, while F2 and F3 only show slight changes after four minutes of observation. F3 (4% of CAE 2% of PEE) has the highest foam volume but it slightly decreases after four minutes. F5 (2% of CAE 4% of PEE) has the lowest foam volume but it has good stability as the volume remains constant over the four minutes of observation. On the other side,

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Table 5: Result on pH test of formulated shampoos (n=3)			
Formulation	Concentration of extract (%)		pH (Mean ± SD)
	P. emblica	*C. asiatica*	
Blank	0	0	8.46± 0.02
F1	0	6	5.93 ± 0.01
F2	1	5	5.54 ± 0.01
F3	2	4	5.10 ± 0.00
F4	3	3	4.78 ± 0.00
F5	4	2	5.13 ± 0.00
F6	5	1	4.83 ± 0.01
F7	6	0	5.75 ± 0.01

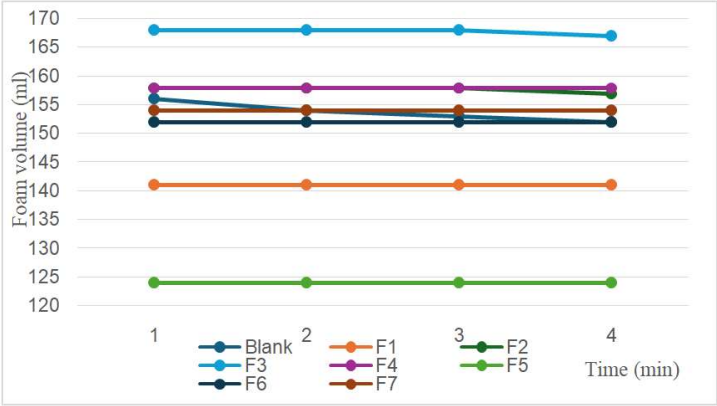

Figure 2: Foam retention profiles of tested shampoos

the blank shampoo formulation also performed good foaming ability, but the foam volume was slowly reducing after two minutes. The foam generated by the prepared shampoos were uniform, compact, and stable.

Based on the graph, it shows that all formulations have good foaming ability and stability. However, in comparison to the blank shampoo, all the shampoo formulations containing the extracts have better foaming stability even though the difference was not significant. In addition, F4 (3% of CAE 3% of PEE) has the second highest foam produced and the foam remains constant over the four minutes of observation. Thus, it indicates that the addition of both extracts into the shampoo formulation does not adversely affect its foaming ability and increase its stability instead. The higher foaming property of formulated shampoos may be due to the sufficient amount of surfactants(23).

Solid content

Table 6 shows the mean value of solid content evaluation conducted on the prepared shampoos. The best range is between 20-30%. The total solid content of shampoo indicates its ability to clean. According to Badi & Khan (16), the best range is between 20-30% because it is easy to apply and rinse out of hair. A shampoo is considered good if the percentages of solid contents are good enough to cause easy

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Table 6: Result on total solid contents of all formulated shampoos (n=3)			
Formulation	Concentration of extract (6)		Solid content (Mean ± SD)
	P. emblica	*C. asiatica*	
Blank	0	0	16.66 ± 1.76
F1	0	6	17.50 ± 2.46
F2	1	5	19.08 ± 3.13
F3	2	4	13.50 ± 0.66
F4	3	3	15.17 ± 1.26
F5	4	2	14.33 ± 1.26
F6	5	1	11.25 ± 0.90
F7	6	0	11.75 ± 1.00

Figure 3: Result of dirt dispersion test

application and removal from the hair. The percentage of total solid contents for all formulated shampoo was found within the range of 11-19% which is less than 20%, the required range. The amount of solid in all of the tested shampoos was deemed insufficient, and the shampoos were expected to be washed out of the hair very quickly(20). However, there is no survey that demonstrates customer satisfaction with adequate number of solid contents in a shampoo. According to observations, the prepared shampoos have a suitable viscosity and are not too watery. All of the excipients used to make the shampoo are water soluble, which could explain why the solid content is insufficient.

Dirt dispersion

One of evaluations that can be done to assess the cleansing action of shampoo is dirt dispersion. The ink should remain in the water portion to be considered as good quality and has better cleansing action. Figure 3 shows the result for dirt dispersion test for all formulated shampoos.All shampoo concentrated the ink in the water portion.

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Table 7: Results on DPPH scavenging activity of different shampoo formulations (n=3)

Formulation	Concentration of extract		Percentage of scavenging activity (Mean ± SD)
	P. emblica	*C. asiatica*	
F1	0	6	27.37 ± 7.12
F2	1	5	58.82 ± 3.20
F3	2	4	92.52 ± 1.43
F4	3	3	84.65 ± 1.12
F5	4	2	88.80 ± 2.01
F6	5	1	77.17 ± 1.88
F7	6	0	90.46 ± 5.13

Shampoos that cause ink to concentrate in the foam are considered low-quality because ink or dirt that remains in the foam is difficult to rinse away and re-deposits on the hair (16). All shampoo concentrated the ink in the water portion, ensuring adequate cleaning potential and actual effectiveness. This might be due to the samenumber of surfactants which act as a cleaning agent in all the seven formulations. Therefore, the prepared formulations are satisfactory.

Antioxidant study

Shampoo formulations were prepared containing combination of *C. asiatica* and *P. emblica* extract of different concentrations and their antioxidant activity were determined by using DPPH method. UV spectroscopy was used to measure the percentage of scavenging effect produced by the formulated shampoos with absorbance measured at 520 nm (17). Table 7 shows the mean percentage of scavenging activity of the tested shampoos.

Based on the result, F1 (6% of CAE) inhibits 27.4% of DPPH which indicates that the single shampoo formulation containing *C. asiatica* extract demonstrated low antioxidant properties. F7 (6% of PEE) inhibits 90.5% of DPPH which indicates that the single shampoo formulation containing *P. emblica* extract shows a high antioxidant activity compared to other formulations. However, the percentage of inhibition of the shampoo combination F3 (4% of CEA and 2% PEE) is 92.5% which is the highest among all tested shampoos. In comparison to ascorbic acid (100µg/ml), which inhibits 99.28% DPPH, this result demonstrates that the combination of both extracts in the ratio of 2% of *P. emblica* and 4% of *C. asiatica* extract in shampoo produces high antioxidant activity. Compared to F1 (6% of CAE), other combination formulation of shampoo also demonstrated high DPPH scavenging activity. Thus, it shows that the shampoo formulation containing combination of both extracts are better than CAE alone and F3 are the highest among all formulated shampoo.

In shampoo containing combination of extracts, different ratios of the extracts produced different antioxidant properties. This is supported by previous research conducted by Joshi *et al*. (17) where the ratio of betel leaves: guava leaves extract of 1:3 g possessed the highest antioxidant properties. This present study shows that F3 inhibited the highest percentage of DPPH compared to other combination formulations, F2, F4, F5 and F6 and comparable to ascorbic acid standard.However, there are no studies that investigate the effect of the combination of these extracts on antioxidant activity and limited antioxidant studies conducted on shampoo formulations. Previous research concentrated solely on plant extracts. Thus, this study has shown that the combination of *P. emblica* and *C. asiatica* extracts in a shampoo produce high antioxidant effects compared to their single formulations.

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Conclusion

The present study aimed to formulate halal hair growth promoting shampoo containing combination of *C. asiatica* and *P. emblica*. All the ingredients used in this study are considered halal based on the origin of each ingredient. Physical appearance, pH, foaming ability and stability, solid content, and dirt dispersion of the shampoo combination were evaluated and compared to the blank shampoo and their single formulations. The results show that the shampoo combinations were not significantly different to the blank and single formulations, instead it has better foam stability. Based on the antioxidant study using DPPH method, F3 (4% of CAE 2% of PEE) shows the highest percentage of scavenging activity compared to other formulations. As a result, the findings of this study have provided the basic information on the feasibility of formulating a halal hair growth promoting shampoo containing combination of *C. asiatica* and *P. emblica* extracts. The blank shampoo formulation produced ideal physicochemical properties and it was not adversely affected by the addition of both extracts. The combination of shampoo (F3) demonstrated high antioxidant properties comparable to the ascorbic acid standard. These findings are important as the combination formulation is not currently available in the market yet and has the potential to be marketed in the future.

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Suppression of Hotair Gene in Small-Cell Lung Cancer Effect The Differentially Expressed Genes and Inhibit Progression of the Disease

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Abstract

HOTAIR gene is a long non-coding RNA (lncRNA) that regulates the differentially expressed genes causing intermediate malignant respiratory disorders like small-cell lung carcinoma (SCLC). Thus, HOTAIR gene can be a predictor for chemotherapy response and therapeutic target against chemoresistance in SCLC. In this study, SCLC and HOTAIR suppressed genes were compared with normal lung tissue samples (control) to identify the differentially expressed genes (DEGs). The number of significant DEGs in both SCLC and HOTAIR suppressed vs control analyses were 4906 and 255 respectively with a cutoff value of ($q \leq 0.05$). Among the DEGs, 48 genes were expressed in both SCLC and HOTAIR suppressed conditions with log2 fold change <2. Eight genes (n=8) (ASAH1, CENPE, FABP5, ACOXL, CTSB, RAB11FIP1, VEGFA, JUN) were significantly expressed ($p<0.05$) in both conditions. CENPE, VEGFA and JUN were lowly expressed in SCLC but highly expressed in HOTAIR suppressed condition. Enrichment analysis on the selected genes using Kyoto Encyclopedia of Genes and Genomes (KEGG) tool shows the cell adhesion and cell cycle pathways were significantly activated in both SCLC and HOTAIR suppressed conditions. About 58 reactome pathways were identified and the post translational protein modification pathway was activated in SCLC while the innate immune system pathway in HOTAIR suppressed gene. These potential HOTAIR suppressed DEGs are CENPE, VEGFA and JUN and they could serve as potential biomarkers for cancer drug development. However, further studies are required to develop the protein interaction model of the enriched genes to analyze the correlation and insight of the therapeutic intervention.

Keywords: Small-cell lung cancer, HOTAIR gene, differentially expressed gene, pathway analysis, cellular mechanism

Introduction

Lung cancer is the leading cause of cancer death among men and second leading cause of cancer death among women (1). There is a slow advancement in survival rate of lung cancer due to late-stage diagnosis. Small-cell lung cancer (SCLC) is difficult to investigate clinically owing to a paucity of substantive tumourspecimens. SCLC is grouped with tumors that display neuroendocrine differentiation. It is common for patients of small-cell carcinoma having metastatic disease and most of them may have relapse within first 2 years after undergoing first treatment. The 2-year survival rate is less than 10 percent in metastatic patients and this cancer is commonly located in major airways in the

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human body (2). Long non-coding RNA (lncRNA) is a non-protein coding RNA with a length of more than 200 nucleotides. It regulates various biochemical and cellular processes in cancer. The lncRNA mechanism requires clarification to be used as a potential biomarker and therapeutic target as tumor progresses (3).

HOTAIR is a long non-coding RNA. It is a splice and poly-adenylated RNA with 2158 nucleotides and exons (4). Deviation of DNA methylation is also a suitable biomarker for cancer diagnosis, prognosis, and prediction in response to chemotherapeutic drug (5). HOTAIR gene has a higher expression level in tumor tissue than the non-tumor tissue in SCLC. This is due to lymphatic invasion and relapse. A previous study stated that (6), different expression levels of HOTAIR can assist in detecting progression stage of cancer. In addition, it helps to predict the survival rate of individuals where different functional SNPs across HOTAIR locus influences cancer risk (1,6). In recent studies, knockdown of HOTAIR increases cell sensitivity to anti-cancer drug, apoptosis and inhibits growth of tumor by decreasing the cell cycle progression. Thus, HOTAIR can be a predictor for chemotherapy response and therapeutic target against chemoresistance in SCLC (2,3).

Omics technology enables the profiling of genomic, transcriptomic, epigenomic and proteomic that can generate various types of biological data. This technology was involved in the studies, namely analysis of gene or RNA expression, epigenetic, protein abundance and even regulatory mechanism (7). This technology makes it possible for diagnosis and prognosis of diseases such as cancers to identify differentially expressed genes (DEGs). This facilitates the identification of potential biomarkers and drug targets for targeted therapeutic medication or treatment providing more insights on cancer or tumorigenesis mechanism, its immune feedback response, and its metastasis condition. Hence, this study aimed to identify HOTAIR suppressed DEGs which could serve as potential biomarkers for cancer drug development.

Materials and Methods

Data pre-processing

In this study, New Tuxedo protocol was applied to analyze the RNA-seq data. It is faster, uses less computing power and produces more accurate results (8). Differentially expressed genes were fed into gene set enrichment analysis (GSEA) tools to determine gene interactive pathways.

Data description

The RNA-seq datasets used in this study are small-cell lung cancer (SCLC) from patients, human normal lung tissue as control and suppressed HOTAIR gene in lung tissue of human. These datasets were downloaded from sequence read archive (SRA), NBCI in FASTQ format. There are 3 biological replicates for each sample to ensure the quality of data (Table 1).

Quality Control

FastQC tool was used to perform quality control on raw sequence data from high throughput sequencing pipeline. This tool checks sequence quality per base, adapters, and GC (Guanine-Cytosine) content per sequence. CutAdapt tool was used to cut the adapters that were attached to the sequence resulting from the Illumina experiments without the help of FastQC. The quality of the sequence was interpreted using boxplot where if the boxplot falls within green threshold, it indicates good quality (9).

Gene mapping and transcriptome assembly

RNA-seq analysis tool comprised of three categories, which are reading alignments, transcriptome assembly or genome annotation and quantification of gene and transcript (10). In this analysis, the New Tuxedo protocol that uses TopHat and BowTie was used for mapping, aligning

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reads, estimating transcript abundance and analysing differential expression. Functional profiling was performed using high throughput functional enrichment tools such as GSEA or DAVID to determine pathways and functions of enriched genes (11).

Identification of significant differentially expressed genes (DEGs)

RNA-seq has various methods to identify novel genes or transcripts used in genetic engineering, identification of mutations and differential gene expression (DGE). Each RNA-seq experiment requires reference genome mapping, where in this analysis the reference genome hg38.p11 was retrieved from NCBI (https://www.ncbi.nlm.nih.gov/datasets/genome/GCF_000001405.37/). Quantification of expression abundance was done on the mapped reads for gene or transcript, but the read depth varies for different samples and direct comparison of samples could not be accomplished. Normalization was done using the Cufflink sub-tool named CuffDiff, which is embedded with methods such as Reads Per Kilobase per Million mapped reads (RPKM) and Fragments Per Kilobase per Million mapped fragments (FPKM). False Discovery Rate (FDR) in Cufflinks was calculated using Benjamini-Hochberg approach with a cutoff value of 0.05 and fold-change cutoff at 2. Figure 1 shows the pipeline of RNA-seq differential expression gene analysis.

Gene Set Enrichment Analysis (GSEA)

To look at the biological significance of differentially expressed genes, gene set enrichment analysis and pathway analysis were performed to identify all the significant gene ontology, which includes biological

Table 1: RNA-seq datasets summary

Type of Datasets	Experiment ID(GSM) / Individual ID(V)	SRA ID
Small cell lung cancer (SCLC)	GSM1464365	SRX669103
	GSM1464366	SRX669104
	GSM1464367	SRX669105
Normal lung tissue	V133	ERX288531
	V81	ERX288597
	V130	ERX288620
HOTAIR suppressed tissue	GSM3375812	SRX4644228
	GSM3375813	SRX4644229
	GSM3375814	SRX4644230

Source: Sequence Read Archive (2019). Retrieved from https://www.ncbi.nlm.nih.gov/sra)

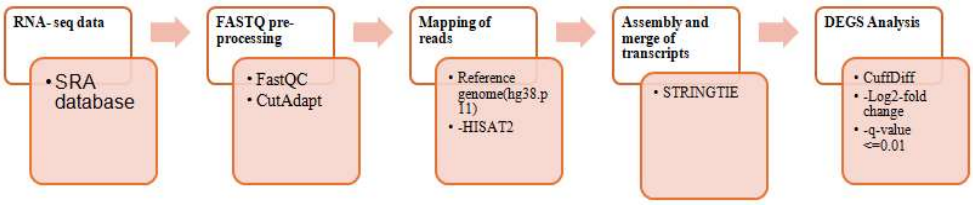

Figure 1: RNA-seq differential expression gene (DEGs) analysis pipeline

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process, cellular components, molecular function and the KEGG and REACTOME pathway (12). Normalized enrichment score (NES) was calculated for gene set based on the degree of over-expressed rank gene list (13). The significant genes were selected based on the cutoff value of q <= 0.01. The selected significant genes were then input into GSEA tools and cross-referenced with the significant gene and annotated genes with 1000 permutations. Databases used in this study are Hallmark gene sets, REACTOME, and Kyoto Encyclopedia of Genes and Genomes (KEGG). Hallmark gene sets reduced the redundancy in gene sets cross referencing results. Pathway with adjusted p-value ($p<0.05$) was generated.

Results and Discussion

RNA-sequencing is popular mainly in cancer research. This is due to its ability to find biomarkers for therapeutic drug target design specialized for each patient (11). RNA-sequencing is more widely used as it can detect novel genes or transcripts and measure level of gene expression by abundance estimation of log2-fold change (14,15). In this study, the transcriptome reads were mapped with the reference genome from NCBI (hg38.p11), which was built using HISAT2 and assembled using STRINGTIE tool. Figure 2 shows the alignment rate of each sample after being mapped to reference genome. Alignment rate more than 60 percent indicates that it has high reproducibility (9) and our data shown in Figure 2 exceeded the threshold of good alignment rate.

Initially, the differential expression gene (DEG) analysis was done on lung cancer sample against normal lung tissue to determine significant genes, which playan important role in the diagnosis ofSCLC. Secondly, the HOTAIR suppressed lung sample was analyzed against the normal lung tissue (control) to observe the significant genes, which play an important role in suppressing the important marker for progression of SCLC.

Our results show that SCLC vs normal produces 4906 significant DEG after applying $q < 0.05$ (Figure 3A). Whereas HOTAIR suppressed sample vs normal have 255 significant DEGs after applying $q < 0.05$ (Figure 3B). Similar findings

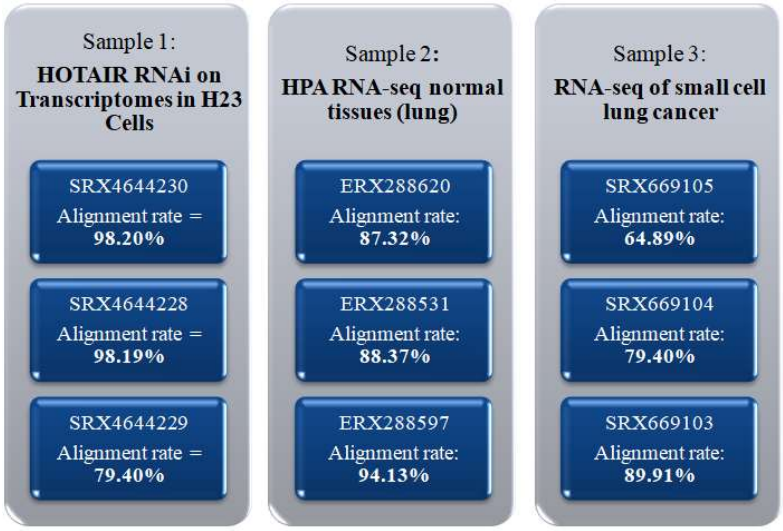

Figure 2: Alignment summary using HISAT2 for each sample. Reference genome used was hg38.p11

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reported (16) where lesser DEGs identified in HOTAIR suppressed sample. This suppression reduces the SCLC cell proliferation in the patient. Among the 255 significant genes identified in HOTAIR suppressed sample vs normal, 80 genes were upregulated (log2FC >= 2), and 78 genes were downregulated (log2FC <= -2). On the other hand, 101 genes were upregulated and 549 were downregulated in SCLC vs normal.

The heatmap of SCLC vs normal shows that the top 50 significant genes have the highest expression level of 3 (Figure 4). In Figure 5, heatmap of HOTAIR suppressed vs normal shows the top 50 highest expression level in only 2.5, which is lesser than SCLC vs normal. The identified DEGs were subjected to KEGG and REACTOME pathway enrichment and Gene Ontology (GO) analysis with False Discovery Rate (FDR) q <= 0.05. We retrieved the top 25 GO enriched pathways (Table 2); 17 belong to biological process domains, 3 from cellular component and 5 from molecular function. This indicates that in the condition of SCLC vs normal, the most affected domain is the biological process of the human body (14). In addition, SCLC causes more disruption in the biological process of the patient as tumor cells may change the functionality of the genes.

The computed enrichment analysis yielded 43 enriched KEGG pathways in SCLC vs normal sample and the top 10 pathways were depicted in Figure 6. KEGG Oxidative Phosphorylation pathway has the highest number of enriched genes. Oxidative phosphorylation pathway is providing the necessity of the tumor cells to carry out proliferation in the form of nutrient or energy (7). The genes which are involved in this pathway are ATP5PO and ATP6V1F.

On the other hand, the computed enrichment analysis yielded 50 enriched REACTOME pathways from this sample. Ranked by NES value for most enriched pathway, the top 10 pathways are selected as shown in Figure 7. REACTOME Post-Translational Protein Modification has the highest number of enriched genes, which is 2. Post-translational protein modification pathway involves biochemical modification that occurs in one or more amino acids after protein being translated (5). The genes involved in this pathway are SUMF2 and YKT6.

The identified DEGs in HOTAIR suppressed vs normal were subjected for enrichment analysis and observedthar 139 GO terms in correlation to the significant genes in this sample. After selecting the top 25 GO enriched pathways, it was observed

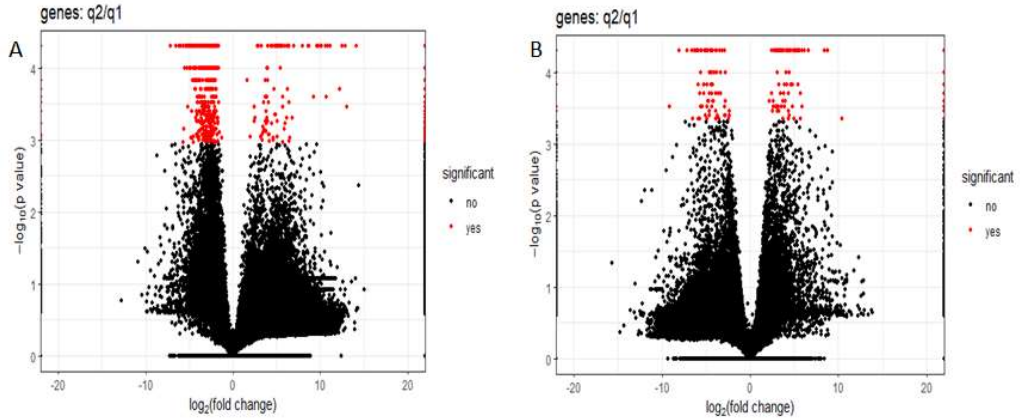

Figure 3A: Volcano plot of SCLC against normal; **3B**: Volcano plot of HOTAIR suppressed against normal

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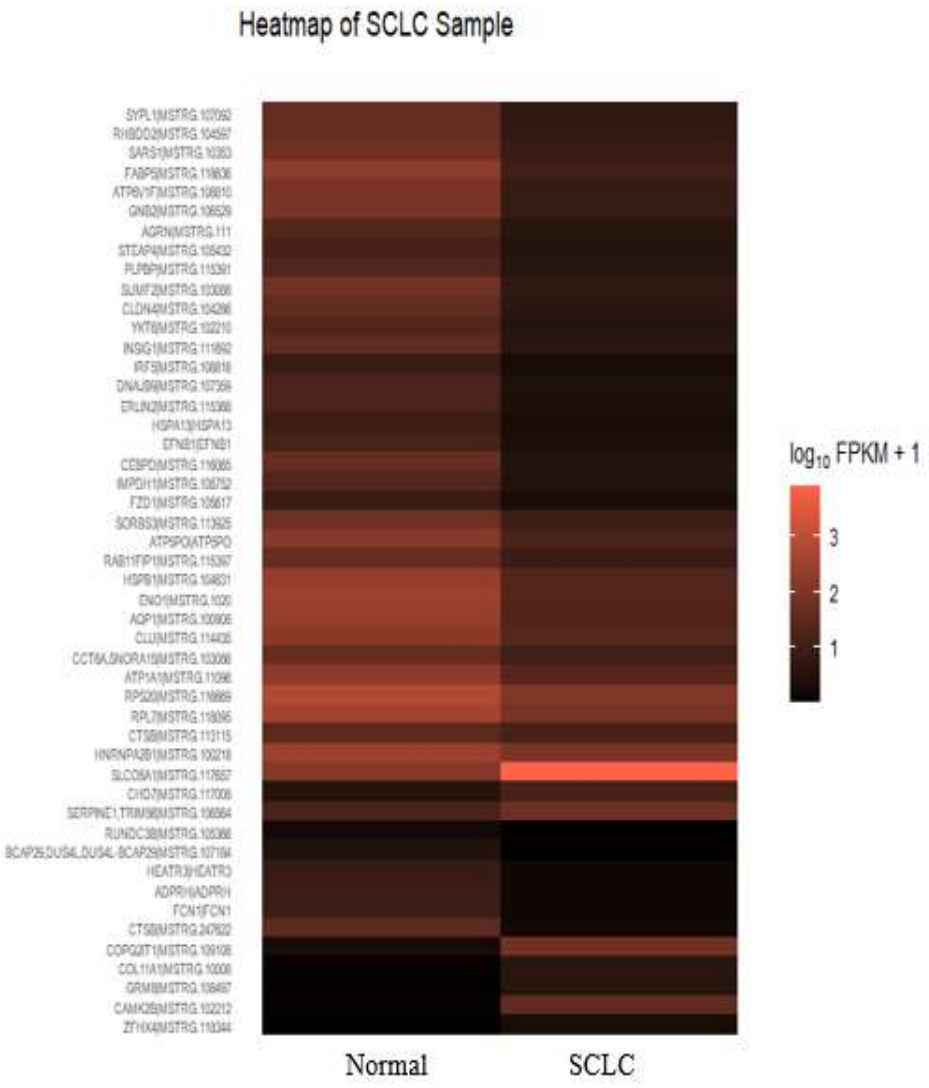

Figure 4: Heatmap of top 50 DEGSs in SCLC vs normal sample (control). Hierarchical clustering of each gene shown in y-axis. Each row represents a gene name, and expression level based on log10FPKM in color key form. Black shows lowest level of FPKM <= 1 and lightest shade of red shows highest level of FPKM >=3 as this sample has very high expression level thus the FPKM is high

that 15 are from biological process domain, 7 are from cellular component and 3 from molecular function (Table 3). The most enriched GO pathway in this sample is GO cell cycle. There were 29 enriched genes in this pathway, which are NCAPG2, NEK2, JUN, FOXM1, MUC1, MAPK13, STIL, TOP2A, CCND2, KIF11, CENPE, IQGAP3,

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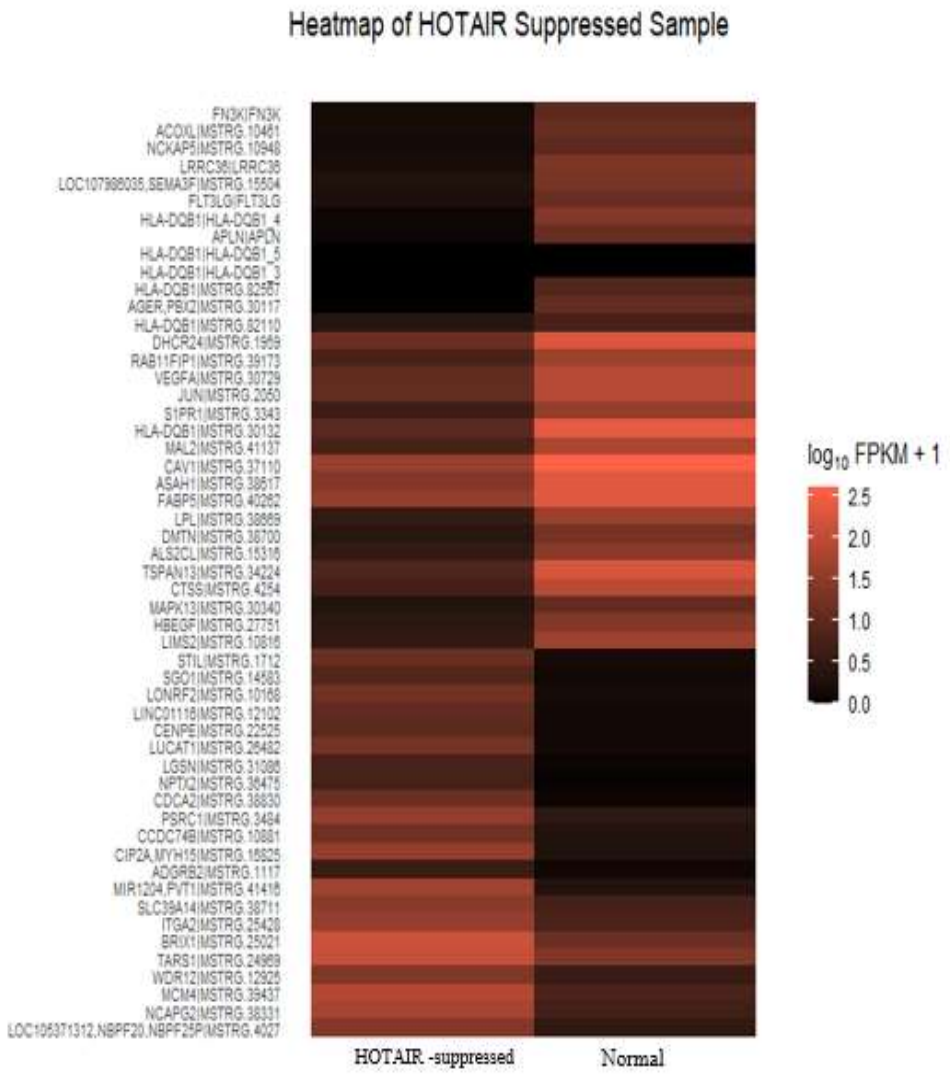

Figure 5: Heatmap of HOTAIR suppressed vs normal sample(control). Hierarchical clustering of each gene shown in y-axis. Each row represents a gene name, and expression level based on log_{10}FPKM in color key form. Black shows lowest level of FPKM <= 1 and lightest shade of red shows highest level of FPKM >=2.5 as this sample has very high expression level thus the FPKM is high

CDCA3, RAD51, TIMELESS, E2F7, SUSD2, FLT3LG, HELLS, CDCA2, PSRC1, SGO1, MCM4, KIF18B, POLE2, MKI67, DHCR24, PPP1R15A and FGFR2. These genes expression level is elevated during knockdown of HOTAIR in which the aid in suppressing development of cancer cell in small cell lung cancer (4).These genes are

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Table 2: Summary of 25 most enriched GO pathways in SCLC vs normal sample

GO Pathway	GO Domain	Enriched Gene Name
GO_CATALYTIC_COMPLEX	Cellular component	ENO1, HNRNPA2B1, HSPB1, RHBDD2
GO_NUCLEAR_TRANSPORT	Biological process	HEATR3, HNRNPA2B1
GO_PASSIVE_TRANSMEMBRANE_TRANSPORTER_ACTIVITY	Molecular function	AQP1, ATP5PO, CLDN4
GO_CELLULAR_RESPONSE_TO_OXYGEN_LEVELS	Biological process	AQP1, ENO1
GO_CATION_CHANNEL_ACTIVITY	Molecular function	AQP1, ATP5PO
GO_POSITIVE_REGULATION_OF_SECRETION	Biological process	AQP1, FCN1
GO_POSITIVE_REGULATION_OF_CELL_POPULATION_PROLIFERATION	Biological process	AQP1, EFNB1
GO_ATP_METABOLIC_PROCESS	Biological process	ENO1, ATP5PO
GO_ATP_BIOSYNTHETIC_PROCESS	Biological process	ENO1, ATP5PO
GO_REGULATION_OF_BODY_FLUID_LEVELS	Biological process	AQP1, HSPB1, CLDN4
GO_CELL_ADHESION_MOLECULE_BINDING	Molecular function	ENO1, YKT6
GO_RESPONSE_TO_OXYGEN_LEVELS	Biological process	AQP1, ENO1
GO_MEMBRANE_PROTEIN_COMPLEX	Cellular component	ATP1A1, YKT6, RHBDD2, INSIG1, ATP5PO, ATP6V1F, CLDN4
GO_RESPONSE_TO_DRUG	Biological process	AQP1, FZD1, ATP1A1, IRF5, AGRN, CLDN4
GO_MAGNESIUM_ION_BINDING	Molecular function	ENO1, ADPRH
GO_REGULATION_OF_VASCULATURE_DEVELOPMENT	Biological process	AQP1, SARS1, HSPB1
GO_CELL_CELL_ADHESION	Biological process	EFNB1, HSPB1, CLDN4
GO_GENERATION_OF_PRECURSOR_METABOLITES_AND_ENERGY	Biological process	ENO1, STEAP4, ATP5PO
GO_CADHERIN_BINDING	Molecular function	ENO1, YKT6
GO_RESPONSE_TO_OXYGEN_CONTAINING_COMPOUND	Biological process	CLU, AQP1, ATP1A1, INSIG1, IRF5, AGRN, ATP6V1F, CLDN4
GO_REGULATION_OF_ANATOMICAL_STRUCTURE_MORPHOGENESIS	Biological process	AQP1, SARS1, FZD1, CAMK2B, HSPB1, CLDN4
GO_GOLGI_MEMBRANE	Cellular component	STEAP4, YKT6, RHBDD2
GO_RESPONSE_TO_OXIDATIVE_STRESS	Biological process	AQP1, FZD1, HSPB1
GO_CELLULAR_RESPONSE_TO_CHEMICAL_STRESS	Biological process	AQP1, FZD1, HSPB1
GO_REGULATION_OF_PEPTIDASE_ACTIVITY	Biological process	AQP1, CLDN4

The ranking of most enriched pathway determined by Negative normalized Enrichment Score (NES) value. The higher the value of NES, the more enriched the pathway is in the sample. This table is arranged in the order of the most enriched pathway from the top.

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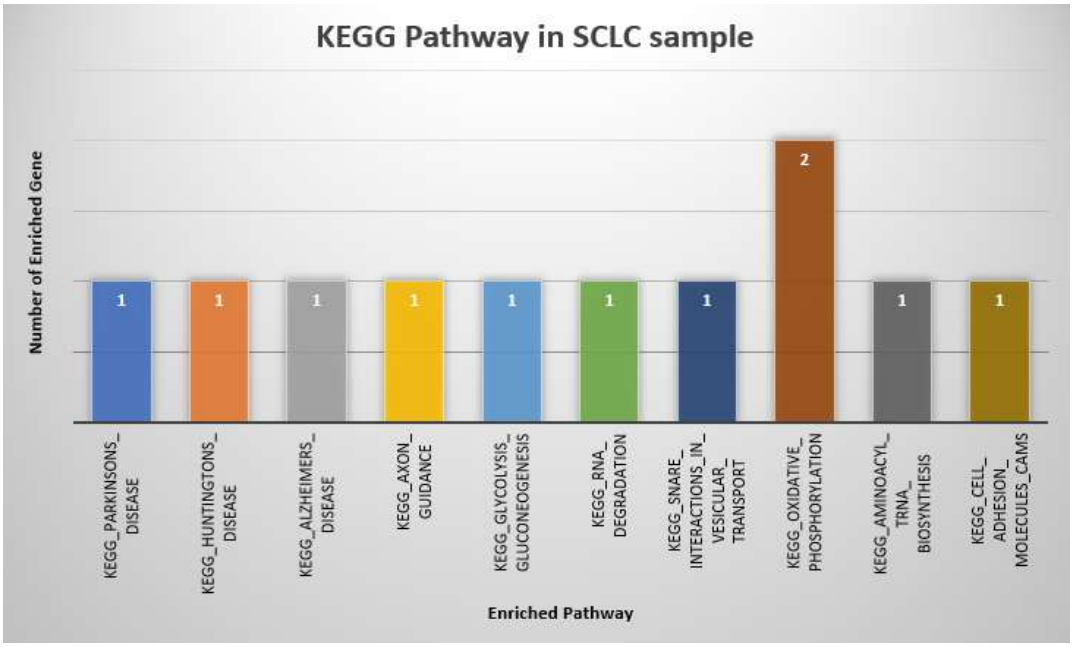

Figure 6: Enriched KEGG pathway in small cell lung cancer sample against normal sample. This bar chart shows the top 10 KEGG pathway out of 43 pathways

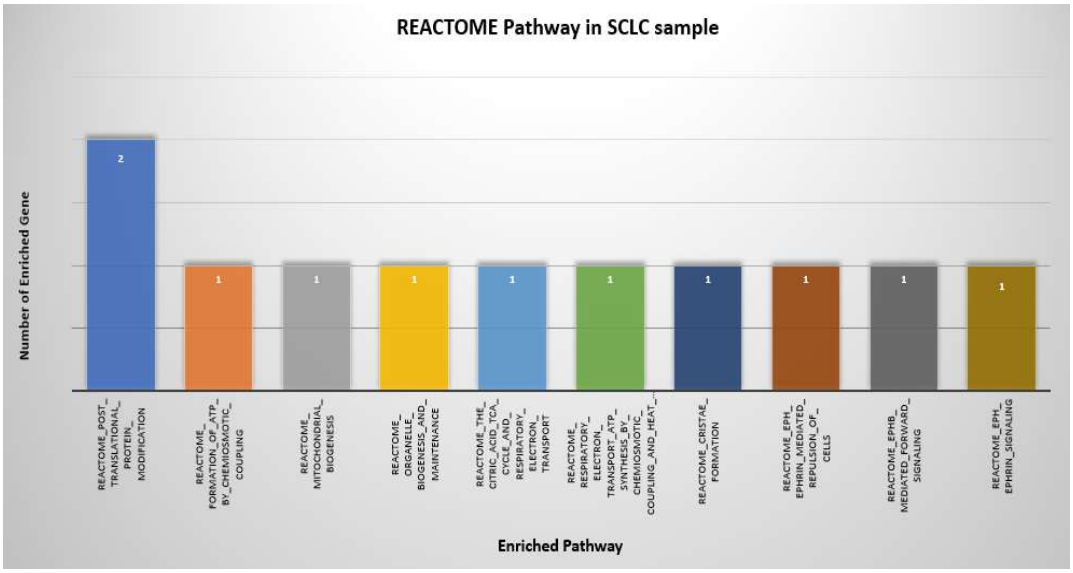

Figure 7: Enriched REACTOME pathway in small cell lung cancer sample vs normal sample.This bar chart shows only the top 10 REACTOME pathways out of 50 pathways

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Table 3: Summary of 25 most enriched GO pathways in HOTAIR suppressed vs normal sample

GO Pathway	GO Domain	Enriched Gene Name
GO_MITOTIC_CELL_CYCLE	Biological process	RAD51, E2F7. NEK2, FOXM1, PSRC1, SGO1, MUC1, MCM4, KIF18B, STIL, TOP2A, CCND2, POLE2, MKI67. KIF11, CENPE, IQGAP3
GO_ORGANELLE_FISSION	Biological process	KIF18B, TOP2A, RAD51, MKI67, KIF11, CENPE, NEK2, PSRC1, SGO1
GO_CELL_CYCLE	Biological process	NCAPG2, NEK2, JUN, FOXM1, MUC1, MAPK13, STIL, TOP2A, CCND2, KIF11, CENPE, IQGAP3, CDCA3, RAD51, TIMELESS, E2F7, SUSD2, FLT3LG, HELLS, CDCA2, PSRC1, SGO1, MCM4, KIF18B, POLE2, MKI67, DHCR24, PPP1R15A, FGFR2
GO_CHROMOSOME_ORGANIZATION	Biological process	VEGFA, RAD51, NCAPG2, NEK2, HELLS, PSRC1, SGO1, MUC1, MCM4, KIF18B, TOP2A, POLE2, MKI67, CENPE
GO_CELL_CYCLE_PROCESS	Biological process	RAD51, TIMELESS, E2F7, SUSD2, NEK2, FOXM1, PSRC1, SGO1, MUC1, MCM4, KIF18B, STIL, TOP2A, CCND2, POLE2, MKI67, KIF11, CENPE, DHCR24, IQGAP3, PPP1R15A
GO_CHROMOSOME_SEGREGATION	Biological process	KIF18B, TOP2A, MKI67, CENPE, NEK2, CDCA2, PSRC1, SGO1
GO_MITOTIC_NUCLEAR_DIVISION	Biological process	KIF18B, MKI67, KIF11, CENPE, NEK2, PSRC1, SGO1
GO_MICROTUBULE_BASED_PROCESS	Biological process	KIF18B, STIL, NCKAP5, KIF11, CENPE, NEK2, WDR35, TRIM46, PSRC1, SGO1
GO_RNA_BINDING	Molecular function	TOP2A, TARS1, MKI67, KPNA2, BRIX1, PDCD11, NQO1, JUN, RPS18

(*Contd.*)

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Table 3: Summary of 25 most enriched GO pathways in HOTAIR suppressed vs normal sample (*Contd.*)

GO Pathway	GO Domain	Enriched Gene Name
GO_CONDENSED_CHROMOSOME	Cellular component	TOP2A, RAD51, MKI67, CENPE, NCAPG2, NEK2, SGO1
GO_MICROTUBULE_CYTOSKELETON	Cellular component	CTSC, RAD51, NEK2, WDR35, PSRC1, SGO1, KIF18B, STIL, NCKAP5, TOP2A, KIF11, CENPE, ADGRB2
GO_CELL_DIVISION	Biological process	VEGFA, TIMELESS, E2F7, SUSD2, NCAPG2, CAT, NEK2, HELLS, CDCA2, PSRC1, SGO1, KIF18B, TOP2A, CCND2, KIF11, CENPE, FGFR2, CDCA3
GO_SPINDLE	Cellular component	KIF18B, KIF11, CENPE, NEK2, PSRC1, SGO1
GO_DNA_METABOLIC_PROCESS	Biological process	MCM4, ACVRL1, TOP2A, RAD51, TIMELESS, POLE2, FOS, KPNA2, NEK2, HELLS, TIGAR, FOXM1
GO_ATPASE_ACTIVITY_COUPLED	Molecular function	MCM4, KIF18B, TOP2A, RAD51, KIF11, HELLS
GO_MICROTUBULE_CYTOSKELETON_ORGANIZATION	Biological process	KIF18B, STIL, NCKAP5, KIF11, CENPE, NEK2, TRIM46, PSRC1, SGO1
GO_SISTER_CHROMATID_SEGREGATION	Biological process	KIF18B, TOP2A, CENPE, NEK2, PSRC1, SGO1
GO_NUCLEAR_CHROMOSOME_SEGREGATION	Biological process	KIF18B, TOP2A, CENPE, NEK2, PSRC1, SGO1
GO_DNA_REPAIR	Biological process	MCM4, RAD51, POLE2, TIMELESS, TIGAR, FOXM1
GO_CHROMOSOMAL_REGION	Cellular component	MCM4, RAD51, CENPE, NEK2, HELLS, SGO1
GO_NUCLEAR_BODY	Cellular component	KIF18B, RAD51, POLE2, E2F7, MKI67, NCAPG2, SGO1
GO_MICROTUBULE_ORGANIZING_CENTER_ORGANIZATION	Cellular component	STIL, KIF11, NEK2, SGO1

(*Contd.*)

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Table 3: Summary of 25 most enriched GO pathways in HOTAIR suppressed vs normal sample (*Contd.*)

GO Pathway	GO Domain	Enriched Gene Name
GO_CHROMOSOME	Cellular component	ARPC5, RAD51, TIMELESS, E2F7, FOS, NCAPG2, NEK2, JUN, HELLS, CDCA2, FOXM1, SGO1, MUC1, CAPN2, MCM4, TOP2A, CCND2, POLE2, MKI67, CENPE
GO_REGULATION_OF_MICROTUBULE_BASED_PROCESS	Biological process	STIL, KIF11, NEK2, TRIM46, PSRC1
GO_ATPASE_ACTIVITY	Molecular function	MCM4, KIF18B, TOP2A, RAD51, KIF11, CENPE, ABCA3, HELLS

The ranking of most enriched pathway determined by Negative normalized Enrichment Score (NES) value. The higher the value of NES, the more enriched the pathway is in the sample. This table is arranged in the order of the most enriched pathway from the top 25 GO pathways

also involved in the progression of cell cycle that comprises of the 4 phases and replication of genome and segregation of chromosome.

The computed enrichment analysis yielded 2 enriched KEGG pathways;Cell Adhesion Molecule (CAMS) and Viral Myocarditis have the same number of enriched genes. Cell Adhesion Molecule (CAMS) pathway involved in the binding of cell surface with other cell or extracellular matrix as it is a subset of the cell adhesion protein. This pathway aids the regulation of a cell or process. The genes which are involved in this pathway are CNTN1, HLA-DQB1 and HLA-DRA. Our data supported where HLA-DQB1 genes are slightly expressed in HOTAIR suppressed condition and highly expressed in normal condition (Figure 5). Next, enriched KEGG pathway is the Viral Myocarditis pathway, which is involved in inflammation of heart muscle due to viral infection. It may be caused by direct cytopathic effect of virus, a pathologic immune response to persistent virus or autoimmunity triggered by viral infection. The genes involved in this pathway are CNTN1, HLA-DQB1 and HLA-DRA and similarly to Cell Adhesion Molecule (CAMS) pathway genes, in which they are under-expressed in HOTAIR suppressed condition and highly expressed in the normal condition.

From REACTOME pathway library, the computed enrichment results yielded 9 enriched REACTOME pathway in HOTAIR suppressed vs normal sample (Figure 8). REACTOME Innate Immune System pathway has the highest number of enriched genes which is 18. Innate Immune System pathway encompasses in the non-specified part of immunity, which is part of individual biological make-up. The genes involved in this pathway are CTSC, CST3, ARPC5, CTSD, CTSB, CTSS, FABP5, FOS, ASAH1, CAT, CREG1, JUN, LYZ, MUC1, MAPK13 SLC11A1, CD81 and JUP. This pathway refers to the non-specified defense mechanism when an antigen occurs. It includes physical barrier and immune system. The main purpose of knockdown of HOTAIR gene is to suppress SCLC and this pathway is the most enriched as the genes involve in the active anti-tumor immune response (1).

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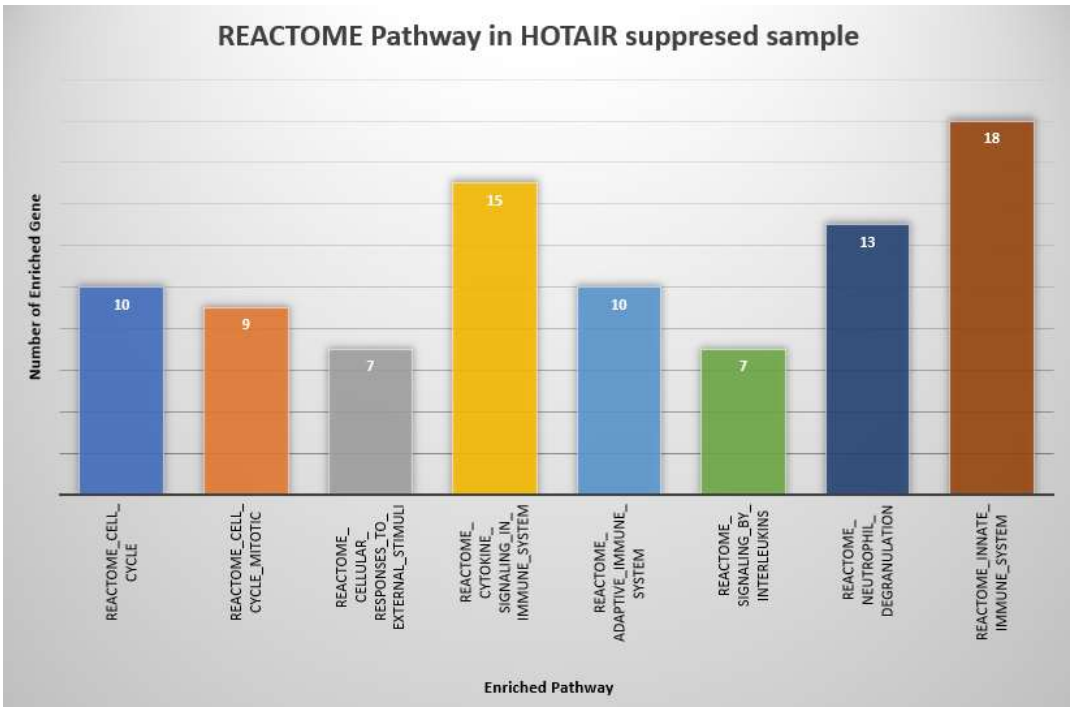

Figure 8: Enriched REACTOME pathway in HOTAIR suppressed sample vs normal sample

Conclusion

SCLC vs normal condition produces 4096 significant differentially expressed genes (DEGs) while HOTAIR suppressed vs normal condition produces 255 significant DEGs. There are apparent differences in total number of DEGs in both conditions because the purpose of knockdown of HOTAIR gene is to suppress small-cell lung cancer progression. Thus, the number of DEGs in a cancer cell is higher when compared to normal cells whereas DEGs of suppressed cancer gene (HOTAIR) is lower than in normal cells.The biological pathway enriched by GSEA shows that SCLC vs normal affects post translational protein modification pathway and for HOTAIR suppressed vs normal effect Innate immune system pathway. In KEGG pathway, the cell adhesion molecule (CAMS) is highly affected because CAMS are tumor suppressant and there is major difference of expression level in both conditions. Seven genes have the tendencyto become potential diagnostic biomarkers for lung cancer. The genes are PLPBP, IRF5, DNAJB9, EFNB1, CEBPD, ENO1 and CLU. In SCLC vs normal sample and HOTAIR suppressed vs normal, there are 8 highly significant genes, which play role in cancer development whereas only 3 genes have significant expression level in HOTAIR suppressed sample. These genes are CENPE, VEGFR and JUN; which serve as possible prognostic biomarkers (17). These genes as potential biomarkers may enhance the effectiveness of drug development for treatment benefits of lung cancer.

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Halal Formulation of Antimicrobial Cream Containing *Melicope ptelefolia* Leaves Extract

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Abstract

Halal pharmaceuticals refer to drugs that are formulated with acceptable ingredients consistent with Islamic principles and conditions. In the pharmaceutical industry, the standard of halal pharmaceuticals is the crucial document that should be followed to standardize the quality and the safety of the products. *Melicope ptelefolia (M. ptelefolia)*, known as tenggek burung was claimed to have many health benefits, including antioxidant, anti-inflammatory and antimicrobial properties. This study aims to formulate halal cream containing *M. ptelefolia* extract. All the ingredients used were evaluated for their halal and safety status based on four supporting documents: Halal Certificate, Certificate of Analysis (CoA), International Nomenclature of Cosmetic Ingredients (INCI) and Material Safety Data Sheet (MSDS). The leaves of *M. ptelefolia* was extracted using methanol solvent and diluted into four different concentrations, 25% w/v, 50% w/v, 75% w/v, and 100% w/v. The extractwas then tested for *S. aureus* and *P. aeruginosa* antimicrobial screening using disk diffusion method. Based on the antimicrobial screening, four types of creams were formulated namely MP0, MP2, MP4 and MP6. It was then evaluated by its color, homogeneity, appearance, phase separation, effect on pH and temperature. The cream formulation was then evaluated for its antimicrobial activities against *S. aureus*. All the *M. ptelefolia* extract concentration demonstrates antimicrobial properties against *S. Aureus* but not *P. aeruginosa*.*M. ptelefolia* extract was then incorporated into the cream formulation with different concentration. Based on the evaluation, all the cream formulations are stable at various temperatures. The results showed that MP6 and storage temperature at 25°C has the highest inhibition zone. In conclusion, stable halal formulation of *M. ptelefolia* as antimicrobial cream was successfully formulated for treatment against *S. aureus*.

Keywords: Halal, *Melicope ptelefolia*, *Staphylococcus aureus,* Antimicrobial cream

Introduction

Halal is an Arabic word that implies "lawful," "permissible" under Islamic law, and it is frequently used in the context of Islamic consumption (Wilson, 2014). Consuming halal items was highlighted in numerous articles of the Qur'an and other sources of Islamic doctrine. Regardless of their geographic or cultural variety, muslims will always adhere to their principles and the Islamic religion. Whenever the concept of halal is mentioned, the concept of Tayyib is expressly mentioned as well. Tayyib means clean, pure, and in accordance with Shari'ah (Alzeer *et al.,* 2020). The Malaysian Standard was created by the National Industrial Standardization Committee and approved by the Department of Standardization Malaysia (DSM) (Azam *et al.,* 2021) are the guidelines that are compulsory to be follow by the manufacturers and distributers in order promote or sell their products in the market.

Pharmaceutical products are rarely halal-certified, especially medicines. As a result, the halal status of certain products remains unknown. Pharmaceutical products

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are made up of active substances and excipients (Aziz *et al.,* 2014). Both the active substance and the excipients must be halal. There must be no non-halal materials used in the manufacturing process (Khan *et al.,* 2013). A pharmaceutical product containing alcohol would be considered halal if there were no adequate alternatives. If any medicine does not have a label and the illness is critical, it can only be used if there are no other options (Halim *et al.,* 2014).

Melicope ptelefolia(*M. ptelefolia*) is a member of the Rutaceae family, and locally known as 'tenggekburung', 'pepauh', 'medangbeberas', 'tapakitik' and 'cabangtiga'. Additionally, *M. ptelefolia* leaves have grown in favor as a traditional fresh vegetable among Malaysians over the years (Abbas*et al.,* 2009). *M. ptelefolia* leaf extract claimed to possess anti-inflammatory, antipyretic, analgesic, antioxidant, and antibacterial effects (Mahadi *et al.,* 2016). 2,4,6-trihydroxy-3geranylacetophenone (tHGA) are the compounds reported to show anti-inflammatory activity (Kabir *et al.,* 2017). Meanwhile, melicolones A and B, isolated from the leaves of *M. ptelefolia*, have been shown to prevent glucose-induced oxidative damage in HUVEC cells (Kabir *et al.,* 2017). Other chemical constituents found in *M. ptelefolia* include p-O-geranylcoumaric acid, various polyprenylated acetophenones and benzylisoquinoline alkaloids (Shaari *et al.,* 2006; Shaari *et al.,* 2011). These secondary metabolites offer the potential of *M. ptelefolia*as an anti-microbial agent, hence this study was conducted to evaluate the effectiveness of *M. ptelefolia* extract incorporated into halal cream formulation against selective bacterial; *Staphylococcus aureus* (*S. aureus*) and *Pseudomonas aeruginosa.*

Materials and Methods

Source of *Melicope ptelefolia*

The matured leaves of *M. ptelefolia* was chosen in this study. 1kg of matured leaves of *M. ptelefolia* were collected from the Institute of Bioscience, University Putra Malaysia (UPM), Serdang. The authentication of the plant was carried out by a qualified botanist from the faculty of Forestry, UPM where the vouchers (KM 0035/22) were obtained.

Identification of halal critical ingredient

Stearic acid has been identified to be the critical ingredient in formulation. Stearic acid was bought from Take It GlobalSdnBhd is halal certified by Jabatan Hal Ehwal Agama Islam Pulau Pinang that is recognized by JAKIM.Other ingredientfor cream formulation was checked via several documentsinclude Certificate of Analysis (CoA), International Nomenclature of Cosmetic Ingredients (INCI) and Material Safety Data Sheet (MSDS) to ensure the safety and the sources of ingredients was plant based.

Melicope ptelefolia methanolic extract

The extraction method was modified from Johari*et al.,* (2011). 1kg of freshly collected matured leaves of *M. ptelefolia* were cleaned, weighed and oven-dried for 48 hours at 40°C. The dried leaves were blended into a fine powder using an electrical blender. 50 g powder was extracted with 250 ml of methanol in five separate batches where the ratio of solvent to sample is 5:1. The macerated mixture was allowed to stand for 24 hours to ensure that all solvent and sample were completely homogenized. The macerated mixture was then filtered, concentrated and evaporated using a rotary evaporator under controlled temperature and reduced pressure. The resultant extract was then stored in a refrigerator at -20°C prior to use. Percentage yield of the extract was calculated in this study by using formula as below:

$$\text{Extraction yield (\%)} = \frac{\text{Mass of extract (g)}}{\text{Mass of dry matter (g)}} \text{ x } 100\%$$

Preparation of different concentration of *M. ptelefolia* extract

Four different concentrations of extract 25% v/v, 50% v/v, 75% v/v and 100% v/v were prepared from the concentration

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liquid extract for antimicrobial screening. Sterile distilled water was used as a solvent (Dahlan *et al.,* 2015). 6 mm filter paper discs were then impregnated with the various concentration of *M. ptelefolia*.

Antimicrobial screening of *M. ptelefolia* extract

Methanolic extract of *M.ptelefolia* leaves were evaluated for antimicrobial activity against *Staphylococcus aureus* (*S. aureus*) (ATCC 25923) and *Pseudomonas aeruginosa* (*P. aeruginosa*) (ATCC 9027) using disc diffusion method of various concentrations. Kirby-Bauer Disc Diffusion method was used as the antimicrobial testing protocol (Nassar *et al.,* 2019). *S. aureus and P. aeruginosa* inoculums were prepared using normal saline. 0.5 McFarland standard was used to ensure the number of bacteria in a suspension isequivalent.A 100 µl of inoculum suspension was withdrawn and transferred to the MH agar using the spreading plate technique. The impregnated disc was then appliedon the agar surface. The agar plate was then stored in an incubator of 37°C for 24 hours. The diameter of zones of inhibition was measured after 24 hours. Sterile distilled water was used as control negative, meanwhile gentamicin disk 10 µg used as control positive.

Physical evaluations of halal formulation of cream

The halal formulation of oil-in-water (o/w) emulsion-based cream was modified from Gidwani *et al.* (2010). Table 1 shows the halal cream formulation used.From the antimicrobial screening results, the extract concentration that gives the highest zone of inhibition will be chosen to formulate 60 g of cream with different *M. ptelefolia* extract weight. 2 g, 4 g and 6 g of *M. ptelefolia*extract was incorporated into the halal cream. The successfully cream formulated was then transferred into plastic container and labeled. Halal formulation of *M. ptelefolia* cream was

Table 1: Halal cream formulation of *M. ptelefolia*

	Cream with 2 g of *M. ptelefolia* extract (MP2)	Cream with 4 g of *M. ptelefolia* extract (MP4)	Cream with 6 g of *M. ptelefolia* extract (MP6)	Cream without *M. Ptelefolia* extract (MP0)
Components	Amount (g)			
Oily phase:				
Stearic acid	1.50	1.50	1.50	1.50
White beewax	0.90	0.90	0.90	0.90
Stearyl alcohol	3.00	3.00	3.00	3.00
Cetyl alcohol	3.90	3.90	3.90	3.90
Mineral oil	3.00	3.00	3.00	3.00
Aqueous phase:				
Propylene glycol	3.00	3.00	3.00	3.00
Triethanolamine	1.20	1.20	1.20	1.20
Methyl paraben	0.01	0.01	0.01	0.01
Propyl paraben	0.03	0.03	0.03	0.03
M. ptelefolia extract	2.00	4.00	6.00	0.00
Water	41.46	39.46	37.46	43.46
Total	60.00	60.00	60.00	60.00

Halal Formulation of Antimicrobial Cream

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inspected visually for its color, homogeneity, consistency and phase separation (Viswanad *et al.,* 2012).

pH test

Digital pH meter was used to determine the pH of various formulations of the cream. The pH meter was calibrated using standard buffer solution with pH 7 and pH 4.01. 0.5 g of o/w cream was weighed and dissolved in 50 ml of distilled water to obtain an even distribution of cream in the solution. pH measurement of various formulation of halal creams were carried out in triplicate and the average reading was recorded.

Effect of temperature on halal formulation of cream

Halal formulation of *M. ptelefolia* cream was stored in three different temperatures which were 4°C, 25°C and 37°C for one month period. Parameters such as physical characteristics, pH and antimicrobial activity were re-evaluated.

Antimicrobial screening of halal formulation of cream

Halal formulation of *M. ptelefolia* cream were screened for its antimicrobial activity against *S. aureus* by using agar disc diffusion method. 2g, 4g, 6g and blank cream was impregnated with the disc. The antimicrobial activity was evaluated by measuring diameter of "zone of inhibition". *M. ptelefolia* cream were not tested on *P. aeruginosa* as it did not show any antimicrobial activity in antimicrobial screening of *M. ptelefolia* extract.

Statistical analysis

Statistical analysis was performed by using the IBM Statistical Package for the Social Sciences (SPSS) Version 28. One-way ANOVA followed by post-hoc, Tukey's test was conducted to determined significance between groups. p value less than 0.05 was accepted as significant.

Results and Discussion

Preparation of *M. ptelefolia* extract

The percentage of *M.ptelefolia* yield extract is 16% as shown in Table 2. Study reported by Kadum et al., (2019), claimed that 16% of percentage yield is considered as a good average yield for many plants extract, however the optimal yield can vary depending on factors such as the plant species, the extraction method, and the intended use of the extract.Methanol was claimed to show a good solvent for plant extraction (Alo *et al.,* 2012). Plants that contain compounds of antimicrobial properties are reported to be soluble in methanol (Naz *et al.,* 2020). Hence, this study uses methanol as a solvent which in line with previous study reportedthat methanolic extract was very potent and has the strongest antimicrobial activity when compared to ethanol and ethyl acetate(Chauhan *et al.,* 2010).Study conducted by Borges *et al.,* 2020 mentioned that 80% methanol gave the highest extract yield during extraction due to solubility of active ingredients, which have polar character.

Evaluation of antimicrobial activities of *M. ptelefolia* methanolic extract

The concentration liquid of methanolic extract of *M. ptelefolia* was prepared and tested at four different concentrations which is 25% v/v, 50% v/v, 75% v/v and 100% v/v against gram-positive bacteria, *S. aureus* and gram-negative bacteria, *P. aeruginosa*. The

Table 2: Percentage yield of methanolic *M. ptelefolia* extract

Sample	Weight of dry plant before extract (g)	Weight of dry extract after solvents have been remove (g)	Percentage Yield (%)
M.ptelefolia	250.00	40.00	16.00

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different concentration of *M. ptelefolia* was proved to show antimicrobial activity as shown in Table 3.

From the study, all *M. ptelefolia* methanolic extract displayed antimicrobial activity against gram-positive *S. aureus* as shown in Table 3. *M. ptelefolia* 25% v/v has the lowest zone of inhibition (9.39 ± 0.43 mm) while *M. ptelefolia* 100% v/v has the highest zone of inhibition (14.18 ± 0.38 mm) for the extract. However, the positive control showed the highest zone of inhibition with 23.20 ± 0.52 mm compared to all group. According to Zainuddin *et al.,* (2010), the higher concentrations of the extract, the larger amounts of metabolite present andthismay lead to a greater potential in inhibiting the growth of the bacteria. In contrast, the lower concentration of plant extract maycontainless active metabolite hence lower the ability to inhibit the growth of microorganisms.Liu *et al*., (2012) reported that *Melicope patulinervia*, a difference species of *Melicope* originated from China, that belong to samefamily, Rutaceaefound to have phenol and flavonoid in the extract. Moreover, it also possessesanti-oxidant and antimicrobial activities against differences fungi species which include *Penicillium.sp, Oxytetracycline hydrochloride, Fusarium graminearum, Botrytis cinerea, Northern Leaf Blight of Corn, Lecannostictaacicula* and *Rhizoctonia solani.* Flavonoids have been reported to possess the antimicrobial activity against gram-positive bacteria, *S. aureus* and *S. epidermidis*via inhibiting nucleic acid synthesis, block the fatty acid synthesis, and inhibit peptidoglycan synthesis (Yuan *et al.,* 2022; Fialová *et al.,* 2021). Phenolic compounds are a type of molecule that contain one or more phenol units, predominantly derived from plants, although they can also be sourced from bacteria, fungi, and marine organisms. Research has indicated that phenolic and polyphenolic substances possess antimicrobial effects against a broad spectrum of microorganisms including methicillin-resistant *S. aureus* (MRSA) (Ecevit *et al*., 2022). In line with the previous study, the inhibition of *S. aureus*in *M. ptelefolia* methanolic extractmay be due to the present of flavonoid and phenol.

Study conducted by Eliaser *et al*., (2018) led to the discovery of two types of quinoline alkaloids – buchapine and 3-(3-methyl-2-butenyl)-4-[(3-methyl-2-butenyl)oxy]-2(1H)-quinolinone – as well as three furoquinoline alkaloids, known as roxiamines A, B, and C, fromflowers, leaves, and twigs of Melicopelunu-ankenda originated from Malaysia. The study revealed that quinoline alkaloids possess anti-viral activity against human immunodeficiency virus. Consistent with the previous study, Fialová *et al*., (2021) claimed that alkaloids present in the plants produce antimicrobial

Table 3: Zone of inhibition exhibited by various concentration of *M. ptelefolia* against *S. aureus* and *P. aeruginosa*

Concentration (% v/v)	Zone of inhibition ± SD (mm)	
	S. aureus	*P. aeruginosa*
Normal Saline	0 ± 0.00[a]	0 ± 0.00
M. ptelefolia 25	9.39 ± 0.43[b]	0 ± 0.00
M. ptelefolia 50	10.62 ± 0.42[b]	0 ± 0.00
M. ptelefolia 75	13.44 ± 0.45	0 ± 0.00
M. ptelefolia 100	14.18 ± 0.38	0 ± 0.00
Gentamicin 10µg	23.20 ± 0.52[a]	14.65 ± 0.19[a]

Note: ANOVA test with post hoc Tukey's test (P < 0.05) where:[a]Statistically significant when compared with all group at p<0.05; [b] Statistically significant when compared with *M. ptelefolia* 100 at p<0.05

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activity against skin pathogens including *S. aureus*. In this study, no zone of inhibition was observed in gram-negative bacteria, *P. aeruginosa*. Similarly, study conducted by Dahlan *et al*. (2015) claimed that methanolic extract of *M. ptelefolia* has no antimicrobial properties against *P. aeruginosa*. This may be due to the different composition or morphology of the cell wall between gram-positive and gram-negative bacteria. The protective and unique feature that distinguishes gram-negative bacteria and gram-positive is the outer membrane. This outer membrane is the main reason for the resistance because of its hydrophobic properties (Breijyeh *et al*., 2020).

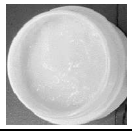
Halal cream formulation and preparation

Halal cream formulation was prepared by incorporating different volume of 100% v/v *M. ptelefolia* extract as an active ingredient. In this study, oil in water (o/w) cream were chosen to be incorporated with *M. ptelefolia* extract. According to Dahlan *et al.* (2015), o/w cream has the ability to release the flavonoids compound of the plant extract which is the constituent of the active compound in *M. ptelefolia*. Another study reported that o/w creams showed the highest ability to release active compounds such as flavonoids compared to other creams such as lipophilic or amphiphilic cream (Sawant *et al.,* 2021). The cream formulated is miscible in with water and skin secretion due to its hydrophilic properties and this results in effective interaction with skin and penetrates more readily through the membrane because of emulsified nature of the skin surface. When the cream is miscible with water and skin secretion, they are easy to be removed from the skin (Bernatoniene *et al.,* 2011).

Physical evaluation

Halal formulation of *M. ptelefolia* cream were characteristically dark greenish in color. Based on this study, the color of the cream increased in intensity as the volume of the *M. ptelefolia* extract increased. Table 4 shows the evaluation of color, homogeneity, appearance and phase separation.From the result, all the halal cream formulation showed

Table 4: Physical evaluation of halal formulation *M. ptelefolia* creams

Formulation	Color	Homogeneity	Appearance	Phase Separation
MP0	White 	Homogenous	Smooth, opaque, greasy on application	No
MP2	Olive green 	Homogenous	Smooth, opaque, greasy on application	No
MP4	Dark olive green 	Homogenous	Smooth, opaque, greasy on application	No
MP6	Dark moss green 	Homogenous	Smooth, opaque, greasy on application	No

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are homogenous cream and no phase separation. The halal cream has the appearance of smooth, opaque and greasy on application for all the formulation. The formulations also are easily removed from the skin when washedwith water. Cream formulation that is not stable will cause the breakdown of the emulsion. The ideal cream should have emollient properties and a smooth texture (Sawant *et al.,* 2021). Another study has shown that cream that is stable are homogenous, almost constant in pH, emollient and easily removed after application (Sharma *et al.,* 2013). Due to emulsified nature of skin surface, drugs formulated as cream are more effectively interact with skin. It is also more readily penetrated through biological membranes (Handali *et al.,* 2011).

Effect on pH and temperature

Halal formulation cream of *M. ptelefolia* were stored at the different storage temperature conditions which is 4°C, 25°C and 37°C for a month. All the cream formulation evaluation of color, homogeneity, appearance and phase separation does not change after a month. This indicates the formulation is stable. Cream that is stable in various temperature conditions will exhibit longer shelf-life. Table 5 and Table 6 show the effect of temperature, pH and zone of inhibition in different temperatures. Based on the study, almost all pH of the formulations increases when the *M. ptelefolia* were added to the bases as the nature of the extract is acidic. pH value after one month for MP2 and MP4 at 37°C is lower than the freshly prepared cream compared to others that increase in pH (Table 7). However, the pH of the skin normally ranges from 4 to 7 (Saptarini *et al.,* 2020). The pH value of the cream ranges from 5.32 to 7.08 was almost similar to the skin's normal pH. Too-alkaline pH preparations will cause scaly skin, whereas too acid pH will cause skin irritation (Viswanad, 2012). This value was acceptable as the pH of the cream will not interfere with normal skin physiology. Studies by Pakzad *et al.,* (2022) stated that there was a slight variation in the pH when the cream stored in different temperature and the rate of

Table 5: pH value of halal formulation *M. ptelefolia* creams after a month of different storage condition

Formulation	pH of freshly prepared cream	pH (mean ± SD)		
		4°C	25°C	37°C
MP0	6.84 ± 0.01	6.91 ± 0.02	7.08 ± 0.03	7.02 ± 0.01
MP2	5.78 ± 0.02	6.57 ± 0.04	5.94 ± 0.01	5.76 ± 0.12
MP4	5.32 ± 0.01	5.73 ± 0.01	5.57 ± 0.13	5.41 ± 0.10
MP6	5.30 ± 0.02	5.57 ± 0.01	5.40 ± 0.01	5.23 ± 0.04

Table 6: Zone of inhibition of halal formulation of *M. ptelefolia* cream *against S. aureus* after a month of different storage condition

Formulation	Inhibition of freshly prepared cream (mm)	Zone of inhibition ± SD (mm)		
		4°C	25°C	37°C
MP0	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00
MP2	6.59 ± 0.08	6.64 ± 0.07	7.57 ± 0.12	7.23 ± 0.12
MP4	7.30 ± 0.36	8.25 ± 0.09	8.33 ± 0.13	8.19 ± 0.10
MP6	8.83 ± 0.33	8.76 ± 0.21	9.21 ± 0.08	9.10 ± 0.04

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Table 7: Zone of inhibition of halal formulation of *M. ptelefolia* cream and control	
Formulation	Zone of Inhibition ± SD (mm)
Negative control	0 ± 0.00
MP2	6.59 ± 0.08
MP4	7.30 ± 0.36
MP6	8.83 ± 0.33
Positive control	24.12 ± 1.77[a]
Note: ANOVA test with post hoc Tukey's test (P < 0.05) where:[a]Statistically significant when compared with all group at p<0.05	

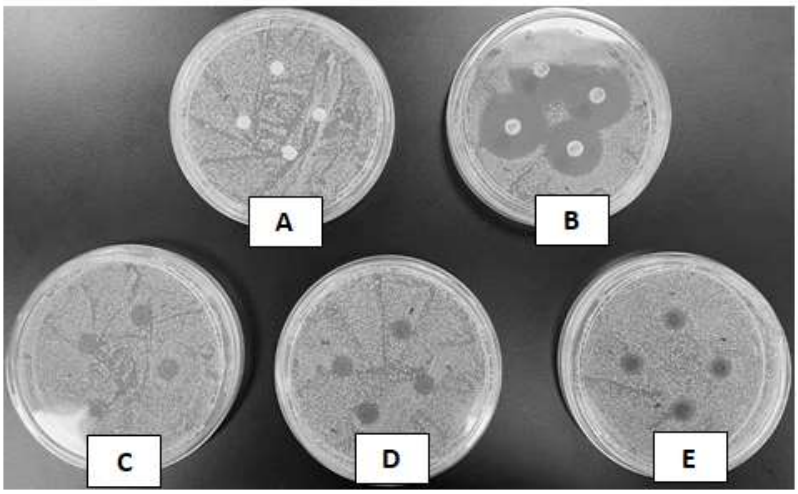

Figure 1: Inhibition zone of *M. ptelefolia* cream against *S. aureus A) MP0 B) Positive control C) MP2 D) MP4 E) MP6*

degradation of cream depends upon two parameters pH and temperature.All types of cream in three conditions showan increasing trend of inhibition. This study found that the halal formulation still exhibits antimicrobial properties after being stored for one monthin three different conditions. This indicates that the halal cream was stable. In comparison between those three conditions, it appears that the best temperature to store cream was in the 25°C condition. This because storage condition of 25°C has the highest zone of inhibition when compared to other storage condition which is 4°C and 37°C. Similar with the pH, study has documented the rate of degradation of cream depends on the temperature (Pakzad *et al.,* 2020). The reduction of antimicrobial activity of natural products by heating may be due to volatilization or the chemical or physical changes that occur during heating (Durairaj *et al.,* 2009).

Evaluation of antimicrobial activity of halal formulation of *M. ptelefolia* cream

Three different formulation of halal *M. ptelefolia* cream were prepared with 2g, 4g and 6g of the 100% v/v extract. The cream was tested on *S. aureus* by using agar disc diffusion method. The zone of inhibition is shown in Table 5. Figure 1 shows the inhibition zone of *M. ptelefolia* cream against *S. aureus.* Based on the studies, all the halal formulation cream containing different

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amount of 100% v/v extract *of M. ptelefolia* shows antimicrobial activity against *S. aureus*. MP6 has the highest inhibition zone compared to others. On the other hand, MP2 that containsthe lowest amount of extract has the lowest inhibition. This result is consistent with earlier antimicrobial test of the extract whereby the higher the amount of extract, the higher the active metabolite that leads to higher inhibition growth of bacteria. According to Dahlan *et al.,* (2015), different amount of *M. ptelefolia* was incorporate into semisolid dosage form or gel formand display edits antimicrobial activity. This show that *M. ptelefolia* extract is a promising source of active ingredient to be added and use for the treatment of infection caused by *S. aureus*.

Conclusion

Methanolic extract of *M. ptelefolia* leaves has shown good antimicrobial activity against *S. aureus*. Concentration of extract plays animportant role in antimicrobial activity. The higher the concentration of extract, the higher the antimicrobial activity. Halal formulation *M. ptelefolia* cream show the similar antimicrobial activity against *S. aureus* as in the extract. Hence, *M. ptelefolia* is a potential active ingredient to be cooperated into pharmaceutical product such as gel or cream and can be used as alternative to treat infection. Further study such as *in-vivo* could be more interesting to conduct to identify the effectiveness of the cream produce.

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Investigating the Menstrual Health Practices and Needs of Rohingya Women Refugees Living in Malaysia

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Abstract

The Rohingya people are the most marginalized minorities in the world. Being female refugees in Malaysia, they are vulnerable to many challenges which may impact their menstrual hygiene. Currently, not much is known about their menstrual health practices and needs in Malaysia in which this knowledge gap needs to be addressed. This study aimed to identify the menstrual health practices and needs among Rohingya women refugees and determine the relationship between their socio-demographic profile and menstrual experiences. A cross-sectional study involving 18 to 55 years old Rohingya women attending QFFD Clinic managed by IMARET in Selayang, Selangor between April to November 2022 was conducted. The MPQ and MPNS-36 were included in the self-administered questionnaire. 70 respondents completed the questionnaire. Majority of respondents had used disposable sanitary pad and able to wash hands every time after changing (n = 65, 92.9%), as well as preferred to use bathroom (n = 50, 71.4%) and household bin (n = 68, 97.1%) as location to change and dispose their menstrual materials when at home. The study had also found that only 47.1% (n = 33) of respondents had positive menstrual experiences with the husband's education level to be statistically significantly associated with respondents' menstrual experiences. This study provides a preliminary data on menstrual health practices and needs of Rohingya women in Malaysia. Nevertheless, they performed poorly in addressing their menstrual experiences. Thus, there is a need to include menstrual health programmes in humanitarian crisis.

Keywords: menstrual health; menstrual management; menstrual needs; refugees; women's health.

Introduction

The Rohingya people are considered among the most persecuted and marginalized minorities in the world as they face forced eviction from their home due to human rights violations done on them (Mohajan, 2018). As a result, Rohingya people were forcibly displaced across neighbouring countries such as Bangladesh, India, Indonesia, Thailand and Malaysia (UNHCR, 2021). After the year of 2010, the number of Rohingya refugees who had come to Malaysia were increasing (Todd *et al*., 2019). According to the United Nations High Commissioner for Refugees (UNHCR) 2022, as many as 184,980 refugees and asylum-seekers had been registered under the organization in Malaysia by the end of July 2022. Despite the large influx of Rohingya refugees entering Malaysia, it does not change the fact that Malaysia remained to be a non-signatory to the 1951 United Nations Convention relating to the Status of Refugees and its 1967 Protocol (Kaur, 2008). This had

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caused the Malaysian government to perceive them as undocumented migrants (Teng & Zalilah, 2011).

Being an undocumented migrant, they faced various challenges such as limited access to working opportunity, formal education, and affordable medical care (Buscher & Heller, 2010; Teng & Zalilah, 2011; Mohd Safwan *et al*., 2020). When it comes to the Malaysian health care system, it is widely known to provide medical care at a low cost through subsidization by the government (Yu *et al*., 2008). Unfortunately, these low-cost medical services are only applicable to Malaysian citizens while foreigners such as refugees who want access to these services are required to pay a high fee (Chuah *et al*., 2018). Their low health literacy and language barriers had also further restricted their access to health care (Chuah *et al*., 2018; Zhooriyati *et al*., 2021). To address this issue, non-governmental organizations (NGOs) such as IMAM Response & Relief Team (IMARET) had started to provide cost-effective medical care, health screening and health education through multiple outreach mobile clinics to the Rohingya refugees residing in Malaysia since June 2015 (IMAM, 2022). Though, a permanent mobile clinic was formed in Selayang, Selangor in March 2021 which is known as the Qatar Fund for Development (QFFD) Clinic (IMAM, 2022). This is to further expand the primary care services such as outpatient treatment, vaccination, counselling and others to the Rohingya refugees on a daily basis (IMAM 2022).

Despite the outreaches from IMARET, it has been reported that Rohingya women have inadequate accessed to services and understanding of sexual and reproductive health (SRH) such as family planning services and contraception (Ahmad Rashidi *et al*., 2022). It has also been noted that they have limited knowledge of and poor practice in managing their menses as well as lack of access to water, sanitation and hygiene (WASH) facilities (Pandit *et al*., 2022). These challenges can be seen in an assessment done by a group of NGOs on Rohingya refugees living in refugee camps in Bangladesh (REACH, 2019). This then could lead to health problems due to SRH needs are not being met as menstrual health is an integral part of a woman's SRH (Glasier *et al*., 2006; Phillips-Howard *et al*., 2018).

Little is known about the menstrual practices and needs of Rohingya refugee women in Malaysia, so researchers conducted a study to address this gap in knowledge. The study aimed to identify the relationship between socio-demographic information and menstrual experiences among these women.

Materials and Methods

This was a cross-sectional study conducted among Rohingya women refugees aged between 18 to 55 years old who had attended QFFD Clinic run by IMARET in Selayang, Selangor from April until November 2022. Respondents were recruited through convenience sampling method. The exclusion criteria for this study were menopause women, individuals who had trouble understanding the questionnaire and spoke in other languages apart from the trained translators. A one-off interview was done by using a self-administered questionnaire with the help of trained translators upon consent. The trained translators were health care workers (HCWs) working at the study site who had basic education and can speak in Malay language. They were trained to understand and familiarize themselves with the questionnaire.

Study tools

The questionnaire contained four main sections which were: (1) socio-demographics, (2) menstrual history, (3) the Menstrual Practices Questionnaire (MPQ) and (4) the Menstrual Practice Needs Scale (MPNS-36). Both the MPQ and MPNS-36 were translated to Malay language. The translated questionnaire had been reviewed

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by a validated academician from University Sains Malaysia (USM). The translated questionnaire was then back-translated to English language and send to the developer of MPQ and MPNS-36. The developer had compared the back-translated questionnaires with the original version and reconciliation was done by incorporating feedback from the developer into the final version of the Malay translated version to provide the most accurate version of the translated questionnaire.

The socio-demographics section included information such as age, marital status, educational level & employment status, household incomes and number of females in a household while the menstrual history section provided information such as age of menarche, duration of period & menstrual cycle, consistency of menstrual cycle, menstrual symptoms and the frequency of disturbance of everyday work & school absenteeism due to menses (Nur Aizati Athirah *et al*., 2019). The MPQ section identified the menstrual hygiene practices of respondents such as the menstrual material (MM) use, its washing and drying, the location of changing, disposal, storage of MM, as well as sanitation practices during their last menstruation (Hennegan *et al*., 2020). For the last section which was the MPNS-36, it measured whether the perceived needs of respondents during their last menstrual period are met or not and evaluate their menstrual experiences by providing information on respondents' perceptions of comfort, satisfaction, adequacy, reliability as well as worries and concerns (Hennegan *et al*., 2020). Version 1 of both MPQ and MPNS-36 were used in the questionnaire.

A reliable translated questionnaire was ensured through a pilot study consisting of 17 respondents and the use of Cronbach's alpha reliability test. The removal of item 7 from the MPNS-36 section was found to contribute to the questionnaire's reliability. In order to assess face validity, the questionnaire was rated by trained translators who reported a moderate level of difficulty in understanding it.

The MPNS-36 was divided into 6 sub-scales. The score from all 6 sub-scales were then summed-up to get the total score which is the menstrual experiences. A higher score would indicate positive experiences. In cases where questions were not applicable to respondents (e.g., respondents did not wash and reuse any MMs, the total score only reflects the mean of relevant questions. Median splits were then used to rank the level of overall menstrual experiences into 2 categories as shown in (Table 1).

Data analysis

The data collected was analysed using Statistical Package for Social Sciences (SPSS) software version 23.0. The socio-demographic, menstrual history, MPQ and MPNS-36 were reported using descriptive statistics where numerical data were presented as mean (SD) or median (IQR) based on their normality distribution while categorical data were presented as frequency and percentage. The relationship between respondents' socio-demographic data and their menstrual experiences were determined using Pearson Chi-Square test and Fisher's Test if the expected count of <5 was more than 20%. For Pearson Chi-Square test, the statistical value, degree of freedom and *p*-value of the test were reported while for Fisher's Test, only the statistical value (if reported by SPSS) and *p*-value were reported. For both tests, the significant level was set at $p<0.05$.

Ethical considerations

Ethical approval and research approval were obtained from Human Research Ethics Committee (HREC) USM [USM/JEPeM/21100678] and IMARET [IMARET/Research/2021/01] respectively.

Table 1: Scoring of menstrual experiences for MPNS-36

Menstrual experiences	Score range
Negative experiences	0.00 to 2.00
Positive experiences	2.01 to 3.00

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Subjects' enrolment throughout the study was voluntary and refusal to participate in the study did not affect participants' medical condition management and care. The respondents were also well-informed regarding the study and assured that their data were kept private and confidential prior to data collection. During the transferring of data into SPSS software, it was entered using index numbers as to remain anonymous. The data presented in the study were presented as grouped data and no individual participants were identified. The data collected were then stored securely by the researchers.

Results and Discussion

Socio-demographic data

Table 2 showed the socio-demographic data of the respondents. A total of 70 respondents completed the questionnaire. The majority of the respondents were aged between 18 to 39 years old (n = 57, 81.4%) with a mean age of 31 ± 8.3 years old. Most of the respondents were married (n = 62, 88.6%), had non-formal education (n = 34, 48.6%) and unemployed (n = 53, 75.7%). Many of the respondents' husbands also had non-formal education (n = 27, 38.6%) but the majority of them were employed (n = 56, 80%). On the other hand, about 75.7% (n = 53) and 81.4% (n = 57) of the respondents' mothers and fathers respectively had no education and majority of them were also unemployed (n = 69, 98.6% for mother; n = 66, 94.3% for father). In terms of household income, a lot of the respondents had a monthly household income within the range of RM 1,001 to RM 1,999 (n = 44, 62.9%). Finally, many of the respondents had 1 to 3 menstruating females in their household (n = 62, 88.6%) with a mean of 2 ± 1.2 females.

The age distribution in this study was comparable with another study done at the same study site where 55.8% of Rohingya women were in the age ranged between 18 to 39 years old (Ahmad Rashidi *et al*., 2022). In Malaysia, unmarried refugees tend to experience poor income generation, social support and network compared to married ones who tend to have a better quality of life (Shaw *et al*., 2018; El Arab & Sagbakken). This is reflected in the result of this study as the majority of the respondents were married.

The high proportion of respondents and their family members who are uneducated can be attributable to the denial of access to school in their hometowns in Myanmar (Amnesty International, 2020). The Malaysian public schools also do not accept refugees' children (Letchamanana, 2013; Palik, 2020). Therefore, the only way of receiving education is through non-formal settings which are mainly organized by UNHCR and NGOs (Letchamanana, 2013; Palik, 2020). Though, this non-formal education faces various challenges such as inadequate facilities and untrained teachers (Letchamanana, 2013; Palik, 2020).

Being refugees, they are also suffering economically due to the absence of formal opportunities to earn a living (Nungsari *et al*., 2020). Thus, many had to engage in informal employment such as working in the construction, manufacturing and agricultural sectors and others (Todd *et al*., 2019). If hired, majority of them would be male refugees in which they could only provide a monthly household income of RM 1,127 (UNHCR, 2016). This explains why the majority of the respondents' husbands are working and were able to achieve a household income within the range of RM 1,001 to RM 1,999. The number of menstruating females in a household in this study could also be reflected in another study which stated majority of the households in refugee settlements had at least one woman of menstruating age (Calderón-Villarreal *et al*., 2022).

Menstrual history data

Table 3 showed the menstrual history data of the respondents. More than half of the respondents had their first period within the age range of 11 to 15 years old

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Table 2: Socio-demographic data of the respondents		
Socio-demographic	Mean (SD)	n (%)
Age (n = 70)	31 years old (8.3)	
18 to 29 years old		36 (51.4)
30 to 39 years old		21 (30.0)
40 to 55 years old		13 (18.6)
Marital status (n = 70)		
Not married		5 (7.1)
Married		62 (88.6)
Divorced		3 (4.3)
Education level (n = 70)		
Formal education		14 (20.0)
Non-formal education		34 (48.6)
No education		22 (31.4)
Employment status (n = 70)		
Full-time		15 (21.4)
Part-time		2 (2.9)
Unemployed		53 (75.7)
Husband’s education level (n = 62)		
Formal education		17 (24.3)
Non-formal education		27 (38.6)
No education		18 (25.7)
Husband’s employment status (n = 62)		
Full-time		52 (74.3)
Part-time		4 (5.7)
Unemployed		6 (8.6)
Mother’s education level (n = 70)		
Formal education		5 (7.1)
Non-formal education		12 (17.1)
No education		53 (75.7)
Mother’s employment status (n = 70)		
Full-time		1 (1.40
Unemployed		69 (98.6)
Father’s education level (n = 70)		
		(*Contd.*)

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Table 2: Socio-demographic data of the respondents (*Contd.*)		
Socio-demographic	Mean (SD)	n (%)
Formal education		3 (4.3)
Non-formal education		10 (14.3)
No education		57 (81.4)
Father's employment status (n = 70)		
Full-time		3 (4.3)
Part-time		1 (1.4)
Unemployed		66 (94.3)
Household income (n = 70)		
≤ RM 1,000		11 (15.7)
RM 1,001 to RM 1,999		44 (62.9)
RM 2,000 to RM 2,999		15 (21.4)
Number of menstruating females in a household (n = 70)	2 (1.2)	
1 to 3 menstruating females		62 (88.6)
4 to 6 menstruating females		8 (11.4)

Table 3: Menstrual history data of the respondents			
Menstrual history items	Mean (SD)	Median (IQR)	n (%)
Menstruation age (n = 70)	14 years old (2.0)		
≤ 10 years old			3 (4.3)
11 to 15 years old			46 (65.7)
≥ 16 years old			15 (21.4)
Duration of period (n = 70)	5 days (2.0)		
1 to 5 days			42 (60.0)
≥ 6 days			28 (40.0)
Duration of menstrual cycle (n = 70)		30 days (2.0)	
≤ 30 days			66 (94.3)
≥ 31 days			4 (5.7)
Menstrual cycle consistency (n = 70)			
Consistent			61 (87.1)
Sometimes consistent			5 (7.1)
Never consistent			4 (5.7)
Menstrual symptoms (n = 125)[a]			
Anger			10 (8.0)
			(*Contd.*)

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Table 3: Menstrual history data of the respondents (*Contd*.)			
Menstrual history items	Mean (SD)	Median (IQR)	n (%)
Anxious			2 (1.6)
Sensitive			1 (0.8)
Insomnia			9 (7.2)
Menstrual pain			59 (47.2)
Chest pain			18 (14.4)
Headache			26 (20.8)
Disturbance of everyday work due to period (n = 70)			
Frequently			10 (14.3)
Occasionally			16 (22.9)
Rarely			10 (14.3)
Never			34 (48.6)
School absenteeism due to period (n = 70)			
Yes due to menstrual pain			16 (22.9)
No			54 (77.1)
[a]n = 125 as respondents can choose more than 1 menstrual symptoms			

(n = 46, 65.7%) in which the period lasted less than 5 days (n = 42, 60.0%). Almost every of the respondents felt that their menstrual cycles lasted less than 30 days (n = 66, 94.3%) and were consistent (n = 61, 87.1%). Out of all the menstrual symptoms listed in the questionnaire, majority of the respondents experienced menstrual pain (n = 59, 47.2%) during their menstrual cycles, followed by headache (n = 26, 20.8%) and chest pain (n = 18, 14.4%). Despite that, only a small percentage of respondents stated that their menstrual symptoms frequently disturbed their everyday work (n = 10, 14.3%). This was also seen in school absenteeism as only 22.9% respondents (n = 16) were absent from school due to menstrual pain when they were a student.

The mean menstruation age and duration of period of respondents in this study were equivalent with one study which stated that puberty age for girls is within 10 to 16 years old with the menstrual flow could range from 3 to 5 days (Thiyagarajan *et al*., 2021). The median of duration of menstrual cycle of respondents in this study was also comparable with another study which mentioned that the average menstrual cycle of most women is 28 days which could also usually last as short as 21 days and as long as 35 days (Rostami Dovom *et al*., 2016).

When compared with another study, menstrual pain also remained the major menstrual symptoms felt during menses (Sadaria *et al*., 2022). The similarity of results further strengthens that menstrual pain is widespread among the general population (Schoep *et al*., 2019). Nevertheless, any menstrual symptoms experienced can affect females' quality of life and reduce their productivity due to poor concentration and motivation felt during the menstrual cycle (Nuranna *et al*., 2018; Geta *et al*., 2020). Though, most females experienced none to mild menstrual symptoms, but some may experience moderate and severe menstrual symptoms which can lead to work and school absenteeism (Eshetu *et al*., 2021; Hennegan *et*

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al., 2021). This suggested that majority of the respondents in this study had experienced mild menstrual symptoms as almost half of the respondents felt that their period had never disturbed their everyday work and as many as 77.1% (n =54) of the respondents had not skipped schools due to menstrual pain.

Menstrual health practices

Figure 1 showed that the majority of them preferred to use disposable sanitary pad as MM (n = 65, 92.9%). Figure 2 showed that most of the respondents were able to change their MMs 3 times or more (n = 45, 64.3%) in which they preferred to change them inside the bathroom (n = 50, 71.4%) when at home as shown in Figure 3. This study has also captured that as many as 92.9% (n = 65) of the respondents had washed their hands every time after changing their MMs as depicted in Figure 4. Figure 5 also illustrated that household bin was the commonest disposal site (n = 68, 97.1%) for disposing their used MMs when at home.

Majority of the respondents in this study has used disposable sanitary pad during their last menstrual cycle despite it being more expensive compared to other MMs (UNICEF, 2019). The reason why they could have afforded it is because their husbands are able to earn more than the monthly wage in

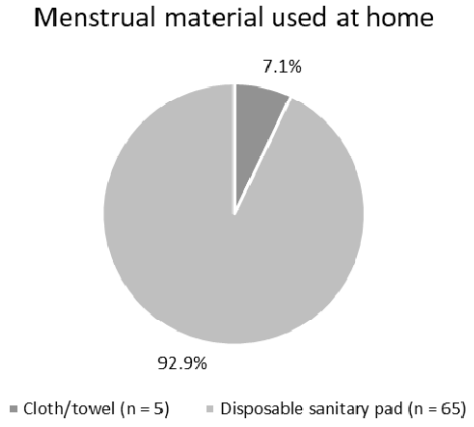

Figure 1: Menstrual material used

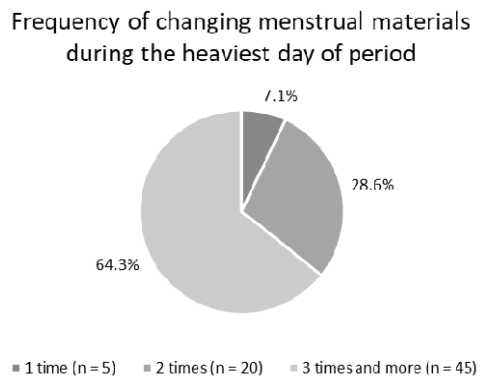

Figure 2: Menstrual materials during the heaviest days of period

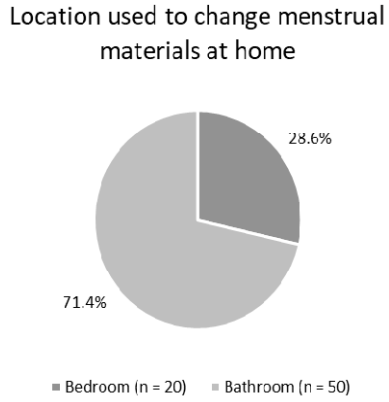

Figure 3: Location to change menstrual materials

Figure 4: Washing hands after changing menstrual materials

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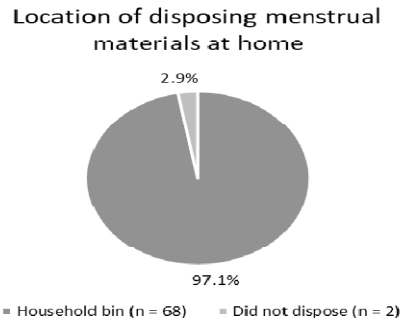

Figure 5: Places of disposing menstrual materials

Malaysia (Nungsari *et al*., 2020).

In Bangladesh, clothes were the major choice of MMs due to the population there had a monthly income lower than Bangladesh's average per capita income in 2019 (Huda *et al*., 2022). When it comes to changing MMs, it is recommended to change them every 6 to 8 hours as failing to do so could lead to infection (Rai *et al*., 2019; Mali & Sudi, 2021).

In Malaysia, Rohingya refugees live in urban settings and not in camps which allow them to have access to nearby shops and buy their MMs when needed (Nungsari *et al*., 2020). Though, in refugee settings, they had to rely on the distribution of sanitary pads by the relevant organization which is infrequent where some had to wait for 2 to 5 months instead of monthly (IOM, 2021; Pandit *et al*., 2022). This explained why only 27.0% of Rohingya girls in Bangladesh were able to change their MMs 3 times and more compared to 64.3% (n = 45) of respondents in this study (Pandit *et al*., 2022).

In this study, bathroom was the major choice of location for changing MMs because Rohingya women in Malaysia have accessed to their own bathrooms and toilets inside their houses. Despite this, a different picture is seen in refugee camps where Rohingya girls had reported challenges in finding spaces for changing their MMs safely and privately (Pandit *et al*., 2022). The challenges included lack of gender segregation facilities, presence of gaps on the walls, poor lighting at night and absence of door locks (IOM, 2021; Pandit *et al*., 2022). This could have affected their MHM as latrines which are safe, clean, lockable, has privacy and good lighting are important factors in managing good menstrual health (Schmitt *et al*., 2021).

It has been described that it is essential to wash hands frequently as a prevention for contracting reproductive tract infections (Ademas *et al*., 2020). Other than that, clean hands are also linked to feeling confidence when managing menses (Hennegan *et al*., 2020). Unfortunately, only 25.7% of Rohingya girls in Bangladesh's refugee camps had washed their hands before and after changing due to the lack of safe and clean water and soap (Pandit *et al*., 2022). This showed that living in urban areas has a higher likelihood of accessing water and can practice hygienic practices as compared to living in refugee camps (Chisty *et al*., 2020). Lastly, it has also been documented that in refugee camps, many women and girls faced difficulty in accessing disposal facility and locating disposal bin (IOM, 2021). Due to the taboo of keeping a bin inside their shelters, many were met with negative remarks such as "shameful" and "dishonourable" for disposing their used MMs in the open by the cleaners (IOM, 2021). This explained why only 6.93% of Rohingya adolescent girls in Bangladesh had used dustbin as the disposal site for their used MMs (Pandit *et al*., 2022). This also shows that living in Malaysia, one is less likely to have trouble finding bins to dispose their used MMs and faced discriminatory remarks.

Menstrual practice needs, concerns and experiences

Table 4 showed the numerical data of sub-scales and total scores of MPNS-36 in which the respondents had a mean total score of 1.99 ± 0.36 with only 47.1% (n = 33) of the respondents had positive menstrual experiences as shown in Figure 6.

This study has shown that the respondents had done poorly in meeting their needs, had higher concerns and lower menstrual experiences when compared to other studies in the same age group (Vural & Varişoğlu, 2021; Hennegan *et al*., 2022). This could be due to lack of access to resources

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Table 4: Numerical data of sub-scales and total scores of MPNS-36		
Sub-scales and total scores of MPNS-36 (n = 70)	Mean	SD
Material and home environment needs	2.23	0.44
Transport and school environment needs	1.37	0.70
Material reliability concerns	2.06	0.94
Disposal insecurity	2.33	0.70
Reuse needs	2.13	0.42
Reuse insecurity	2.00	0.58
Total score (menstrual experiences)	1.99	0.36

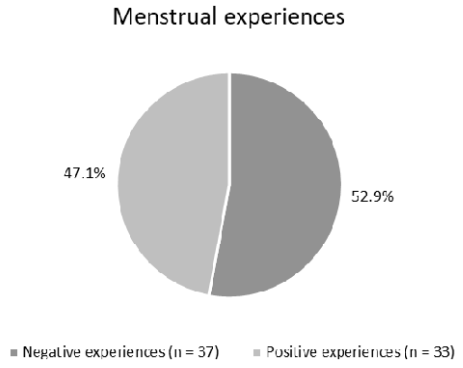

Figure 6: Menstrual experiences

(Hennegan *et al*., 2022). Being a refugee in Malaysia, the respondents in the present study are perceived to be undocumented migrant (Kaur, 2008). Thus, they are unable to get access to formal education and jobs with their movements also being restricted (Teng & Zalilah, 2011; Mohd Safwan *et al*., 2020; Suzarika *et al*., 2020).

In three separate studies which were conducted in India, Ethiopia and Bangladesh, they had shown that females with higher education have better menstrual hygiene management (MHM) and experiences (Bhore & Kumbhar, 2014; Belayneh & Mekuriaw, 2019; Rakhshanda *et al*., 2021). Sufficient knowledge of managing period was also found to be associated with positive menstrual attitudes in females throughout the globes from low- to high-income countries (Munro *et al*., 2021). Most of the Rohingya women in this study, who are uneducated, have poorer menstrual experiences which were found in this study.

Rohingya refugees could only work in the Malaysian informal economy (Todd *et al*., 2019). In the informal economy, there is a lack of enforcing occupational safety regulations and standards where employers are not obligate to provide females employees with the necessary workplace environment which is suitable for their sanitation-related needs (Sommer *et al*., 2016). Some employers may even perceive their sanitation-related needs as unimportant (Sommer *et al*., 2016). Even if there were adequate WASH facilities, they might be prevented from freely using them, such as having to pay before using the sanitation facilities which then increased their incurred costs (Hennegan *et al*., 2020). All of these might also contribute to the reason why most of the respondents in this study are unemployed.

The act of going outside could also pose a risk to the refugees in Malaysia as they are liable to be detained, arrested, and raided (Tasneem *et al*., 2022). According to a report published by the International Federation for Human Rights (FIDH) in 2008, 17,700 refugees had been arrested in 2006 during raids. Most of the raids took place near their living quarters as well as workplaces and despite having proper work permits, most of the refugees were detained (FIDH, 2008). Thus, this prevented them from being able to safely buy their menstrual materials and products without having to face consequences.

Relationship between socio-demographic data and menstrual experiences

Table 5 showed the relationship between various socio-demographic data of

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Table 5: Relationship between socio-demographic data and menstrual experiences

Socio-demographic items	Menstrual experiences, n (%)		Statistic value (df)	*p*-value
	Negative	Positive		
Age (n = 70)				
18 to 29 years old	21 (56.8)	15 (12.1)	5.750 (2)[a]	6.560
30 to 39 years old	13 (35.1)	8 (24.2)		
40 to 55 years old	3 (8.1)	10 (30.3)		
Marital status (n = 70)				
Not married	1 (2.7)	4 (12.1)	4.296[b]	0.107
Married	33 (89.2)	29 (87.9)		
Divorced	3 (8.1)	0 (0.0)		
Education level (n = 70)				
Formal education	9 (24.3)	5 (15.2)	5.724 (2)[a]	0.057
Non-formal education	13 (35.2)	21 (63.6)		
No education	15 (40.5)	7 (21.2)		
Employment status (n = 70)				
Full-time	9 (24.3)	6 (18.2)	0.642[b]	0.784
Part-time	1 (2.7)	1 (3.0)		
Unemployed	27 (73.0)	26 (78.8)		
Husband's education level (n = 62)				
Formal education	14 (42.4)	3 (10.4)	18.827 (2)[a]	0.001*
Non-formal education	6 (18.2)	21 (72.4)		
No education	13 (39.4)	5 (17.2)		
Husband's employment status (n = 62)				
Full-time	25 (75.8)	27 (93.1)	4.197[b]	0.126
Part-time	4 (12.1)	0 (0.0)		
Unemployed	4 (12.1)	2 (6.9)		
Mother's education level (n = 70)				
Formal education	1 (2.7)	4 (12.1)	3.292[b]	0.152
Non-formal education	5 (13.5)	7 (21.2)		
No education	31 (83.8)	22 (66.7)		

(*Contd.*)

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Table 5: Relationship between socio-demographic data and menstrual experiences (*Contd*.)

Socio-demographic items	Menstrual experiences, n (%)		Statistic value (df)	*p*-value
	Negative	Positive		
Mother's employment status (n = 70)				
Full-time	0 (0.0)	1 (3.0)	No statistical value[b]	0.471
Part-time	0 (0.0)	0 (0.0)		
Unemployed	33 (100)	32 (97.0)		
Father's education level (n = 70)				
Formal education	1 (2.7)	2 (6.1)	1.456[b]	0.542
Non-formal education	4 (10.8)	4 (18.2)		
No education	32 (86.5)	25 (75.8)		
Father's employment status (n = 70)				
Full-time	1 (2.7)	2 (6.1)	1.367[b]	0.790
Part-time	1 (2.7)	0 (0.0)		
Unemployed	35 (94.6)	31 (93.9)		
Household income (n = 70)				
≤ RM 1,000	6 (16.2)	5 (15.2)	0.294[b]	0.863
RM 1,001 to RM 1,999	24 (64.9)	20 (60.6)		
RM 2,000 to RM 2,999	7 (18.9)	8 (24.2)		
Number of menstruating females in a household (n = 70)				
1 to 3 menstruating females	35 (94.6)	27 (81.8)	No statistical value[b]	0.136
4 to 6 menstruating females	2 (5.4)	6 (18.2)		

[a]Pearson chi-square
[b]Fisher's exact test
*p-value <0.05 is significant

respondents and their menstrual experiences. This study has found that there was a statistically significant relationship between husband's education level & respondents' menstrual experiences ($p < 0.05$). On the other hand, other socio-demographic data have showed no statistically significant relationships with respondents' menstrual experiences ($p > 0.05$).

When it comes to male responsibility at household level, they are involved in the decision-making on the allocation of household resources (Mahon *et al*., 2015). Thus, their knowledge on menstruation is important as husbands are seen as the main breadwinner of the family (UNFPA, 2022). Though, it was revealed that most of the time, the husbands did not discuss about menstrual issues with their wives and daughters (Mahon *et al*., 2015). There were also instances where the males in the households were ignorant about menstrual products which then caused problems for the females when it came to requesting money to

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buy them (Mahon *et al*., 2015). Thus, it is important to break the stigma by involving men in the discussion of menstruation as males with poor knowledge of menstruation were more likely to endorse cultural myths about menstruation and restrictions on menstruating females which can negatively impact their menstrual experiences (Cheng *et al*., 2007; Mahon *et al*., 2015). When husbands are educated, the study found a higher prevalence of positive menstrual experiences among the respondents (n = 24, 82.8%). In contrast, those who are uneducated had a significantly lower prevalence (n = 5, 17.2%).

Conclusion

In conclusion, it was found out that there were various menstrual health practices of Rohingya women refugees in Malaysia during their last menstrual cycle which differ from those who are living in refugee camps. Nonetheless, Rohingya women refugees performed poorly in addressing their menstruation-related needs, concerns and experiences compared to other studies in the same age group. Thus, there is a need for the agency involved in humanitarian work and NGOs to improve the livelihood of refugees in Malaysia through promoting access to education, work and menstrual health products. For example, they can focus their efforts more on the uneducated and unemployed menstruating women. It is also essential for every stakeholder to work together in implementing menstrual health programmes especially during humanitarian crisis as a way of going forward to combat period poverty. An example of menstrual health programme is by developing and distributing a menstrual health kit which is equipped with basic information on menstrual hygiene practices, disposable sanitary pads, painkillers and others. They could also involve the men in the Rohingya community in the menstrual health programme or workshop as the present study was able to find a relationship between the male education and menstrual experiences experienced by their counterparts.

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Extraction and evaluation of Hemiparasitic shrub of *Scurrula ferruginea* leaves: Phytochemical Analysis, Antioxidant, Antimicrobial and Cytotoxic Activities

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Abstract

Scurrula ferruginea (SF), a tropical obligate hemiparasitic shrub found on branches of dicotyledonous trees in Southeast Asia, has been used by indigenous people as a medicinal herb. This study aimed to evaluate the antioxidant, antibacterial, and lymphocyte proliferation of *SF* extracts and characterize their bioactive compounds.Leaves of *Scurrula ferruginea* were pulverized into powder followed by extraction method. Total phenolic content and total antioxidant activities were measured. Disc diffusion and well diffusion assays to investigate the antibacterial properties of the extracts against *Vibrio parahaemolyticus*, *Staphylococcus epidermidis*, and *Pseudomonas aeruginosa.* The crude extract was shown to contain phenolic compounds and possessed antioxidant abilities. Preliminary investigations revealed the presence of long-chain alcohols, phytol (diterpene alcohol and a constituent of chlorophyll); squalene, (a triterpene and precursor to steroids); and lupeol (a triterpene) which may contribute to the biological activities of this plant. Aqueous extracts of *SF* (2.5 mg/disc) showed the highest inhibition for *Vibrio parahaemolyticus* and *Staphylococcus epidermidis*, but not *Pseudomonas aeruginosa* by disk diffusion. The extracts (particularly organic solvents) were cytotoxic to primary human lymphocytes above 100 µg/ml. There were no effects on lymphocyte proliferation at lower concentrations.

This study evaluates the leaves of ethyl acetate and aqueous fraction of SF showed the properties of potential antibacterial and antioxidative properties against *Staphylococcus epidermidis* and *Vibrio parahaemolyticus*.

Keywords: Antibacterial; Antimicrobial; Cytotoxic; Antioxidants, *Loranthus*

Introduction

Scurrula ferruginea (SF) is a mistletoe within the *Loranthaceae*family. *SF* is a tropical obligatehemiparasitic shrub that grows on branches of a host plant, usually a dicotyledonous tree, such as *Tabebuia pallida*[1]. The rusty appearance of the leaves has earned the plant local names, such as *dalu-dalu*, *dedalu-api,*or *benalu* in Southeast Asian countries [1,2]. Figure 1 shows the flowers and leaves of *SF,* taken offa host plant, *Tabebuia pallida*. This mistletoe without functional roots acquires water and nutrients from the host plant through a highly modified haustorium[3].

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Figure 1: Flowers and leaves of *Scurrula ferruginea* on its stalk

Mistletoes have long been used as medicinal sources to cure many diseases and were found to be useful against cancers, microbes, oxidation and hypertension[4]. An advantage of using plants to treat diseases is its lowered toxicity to the patient with its potential for combating drug-resistant disease [5]. Antibiotic resistance is rampant globally, and there is a need to for new antibiotics that can fight microbes [6]. *SF* has been used as traditional medicine, exerting antimicrobial effects, and antihypertensive effects in rats[1,4]. The crude acetone extract of the leaves, stems and flowers of *SF* contains various phenolic compounds known to have antibacterial effects against *Staphylococcus aureus, Bacillus subtilis, Escherichia coli and Pseudomonas putida*[1,4].

Phytochemical constituents of plants are responsible for their pharmacological activities. Major classes of antimicrobial compounds found in plants include phenols, quinones, flavonoids, and flavanols. A number of these compounds have been shown to be present in *SF* and are thought to be responsible for its antimicrobial effects [7]. The antioxidant activity of various extracts of *SF* have also been studied by several groups. Puneetha *et al.* and Mohsen M *et al*. showed that the plant had a remarkable amount of antioxidant activity[8,9]. *SF* has recently been reported to exert anti-inflammatory effects via nitric oxide generation and certain cytokines such as IL-6, IL-10, IL-1β and TNF-α [10,11]. Additionally, antibacterial studies on *SF* showed that the plant extract moderately inhibited the growth of *Staphylococcus aureus, Bacillus subtilis, Escherichia coli and Pseudomonas putida* [8]. *SF* also exerted apoptotic effect on human breast cancer cell MDA-MB-231. The study suggested that the plant's antioxidant activity, high phenolic and flavonoid content may induce cytotoxicity and apoptosis in breast cancer cells[8].

The anticancer effects of *SF* were also observed with structures of the specific phytochemical compounds elucidated (flavanols including quercetin, quercitrin and 4'-O-acetylquercitrin)[12]. Among the flavanols, quercetin exhibited the most lethal cytotoxic activity with IC_{50} of 35 µM while quercitrin and its acetyl derivatives remained inactive with IC_{50} of 200 µM. The majority of studies evaluated the cytotoxicity of *SF* extracts in immortalized breast cancer cell lines (MDA-MB-231 and MCF-7), inferring that normal healthy cells would not be affected[8,13]. However, there are limited studies on the structures of bioactive compounds involved, or correlated known bioactive phytocompounds with bioactivity.

Here, we aim to investigate the antimicrobial effects of the extracts of *SF* against three bacterial strains, namely, *Staphylococcus epidermidis, Vibrio parahaemolyticus* and *Pseudomonas aeruginosa* and further isolate and characterize the bioactive compounds. Total phenolic content and antioxidant activity and other analytical techniques such as infrared spectroscopy and GC-MS were also utilized to identify and characterize the bioactive

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compounds. In addition, we evaluated the cytotoxicity of these crude extracts against primary human lymphocytes and their effect on lymphocyte proliferation.

Materials and Methods

Chemicals

Various solvents such as methanol, ethyl acetate, hexane/dichloromethane were obtained from Sigma-Aldrich (MO, USA). The chemicals for cell line studies are sodium carbonate, Folin-Ciocalteu reagent, Gallic acid, phosphomolybdenum, sodium phosphate, ammonium molybdate, hydrogen peroxide and ascorbic acid were purchased from Sigma-Aldrich (MO, USA). For the cell culture method were used as per ethics committee approved such as peripheral blood mononuclear cells, fetal calf serumpenicillin/streptomycin and phytohemagglutinin were purchased from Sigma-Aldrich (MO, USA).

Collection, identification and processing of plant material

Aerial parts of *SF* were collected from *Tabebuia pallida* host plant grown along the roadat latitude N 4° 54’ 57.2", Longitude E 114° 56’ 54.0" in February 2017. The plant was separated into different parts: leaves, stems and flowers. The plant was identified and authenticated by theInstitute of Biodiversity and Environmental Research, andcatalog identification was assigned to the submitted samples (*Scurrulla ferruginea*; S01083) for reference. Leaves were cleaned of any extraneous materials using tap water followed by distilled water. They were oven-dried at 50°C and pulverized into powder in a blender. The extracts were prepared separately via four different methods yielding four different types of extracts, namely: crude maceration, methanolSoxhlet, ethyl acetate and aqueous extracts[14].

Preparation of crude methanol maceration extract

50 g of powdered plant material was dissolved in 200 ml of methanol (Merck, Frankfurter, Darmstadt, Germany) for three days at room temperature with several agitations. Precipitate was filtered using filter paper (Sigma-Aldrich, Lesquin, France). The extraction was repeated three times with fresh solvent each time to ensure that the biological products from the plant were released. The filtrates were pooled together and evaporated using a rotary evaporator (Yamato, Tokyo, Japan) in a water bath at 50°C. The yield was a dark green paste, weighing 3.2 g, which was later stored at 4°C[15].

Preparation of aqueous extract

50 g of prepared powder was dissolved in 50 ml distilled water. Using homogeniser (IKA, Germany), the mixture was homogenised for 5 minutes, followed by 2 hours of 10 minutes intervals at room temperature with Ultrasonic Bath (Branson, Missouri, USA) to break the plants’ cell walls for release of the active biological components[16,17]. The sonicated mixture was filtered and stored in sterile falcon tubes at -80° C refrigerator overnight. The solid frozen mixture was freeze-dried for 48 hours, yielding 3 g of light sand-brown fine powder. The extract was stored at 4° C.

Preparation of ethyl acetate extract

50 g of the prepared powder was dissolved in 50 ml of ethyl acetate and 50 ml of distilled water. The mixture was stirred thoroughly for 15-20 minutes using magnetic stirrer (Sigma, Schnelldorf, Germany). The mixture was then left to separate into two layers in a separating funnel, where the top layer was ethyl acetate while the bottom was aqueous. The extraction was repeated three times and the respective layers were pooled together. The cumulative ethyl acetate layer was evaporated till dry using a rotary evaporator (Yamato, Tokyo, Japan) in water bath at 50° C to obtain 1.4 g of dark green to black paste and weighed 1.4 g. The extract was stored at 4° C[18].

Preparation of methanol Soxhlet extract

Methanol Soxhlet extract was prepared using Soxhlet apparatus (Fischer

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Scientific, Loughborough, UK); 200 g of powdered *SF*leaves were weighed and transferred into the cellulose thimble. 2 L of methanol was then poured into the round bottomed flask of the apparatus, to which a few glass beads was added to prevent excessive boiling of the extracting solvent. The apparatus was set up carefully and the heating mantle was set up to the boiling point of methanol, 65°C. Sixteen cycles of distillation were required for complete extraction of the leaves, followed by evaporation using a rotary evaporator (Yamato, Tokyo Japan) in water bath at 50°C which produced 3.6 g of dark green paste (extract). The extract was stored at 4° C[18].

Fractionation via column chromatography

The ethyl acetate extract from the methanol maceration product was column chromatographed on silica gel 60 (70 – 230 mesh) using a graduated solvent system of increasing polarity starting with hexane (100%), followed by a mixture of hexane/ dichloromethane (90:10), dichloromethane/ methanol (1 – 10 % methanol), and finally 100 % ethyl acetate. Five fractions were obtained from this initial fractionation process[19].

ATR-IR and GC-MS analysis

Infrared spectra (IR) were recorded on an attenuated total reflection (ATR) plate attachment on the FTIR spectrophotometer, between 800 and 4000 cm^{-1}.Gas chromatography with mass spectrometry (GC-MS) analyses were conducted for the crude extract and all the five fractions. Samples (crude extract or fractions) were dissolved in liquid chromatography (LC)-grade methanol at 1 mg/mL concentration, and filtered using a 0.2 µm syringe filter into a *GC* vial. The sample was introduced by injection at a flow rate of 1.22 mL/min with an injection volume of 7 µL[20,21].

Total phenolic content (TPC) and total antioxidant capacity

Total phenolic content of the crude extract was analysed using the Folin-Ciocalteu colorimetric method [8]. 20 µL of 1 mg/mL crude extract was mixed with 0.75 mL of 20 % sodium carbonate solution and 0.25 mL of Folin-Ciocalteureagent. The reaction mixture was incubated for 1 h in the dark. The absorbance of the mixture was measured at 765 nm. Standard Gallic acid in the range of 0 – 100 µg/mL was prepared in the same manner and results were expressed as mg gallic acid equivalent (GAE) per gram of extract.Total antioxidant capacity of the crude extract was analysed using the phosphomolybdenum method [9], where 0.3 mL of the extract 100 – 1000 µg/mL (PPM) was mixed with 3 mL of phosphomolybdenumreagent solution (1 mL of 0.6 M sulphuric acid, 1 mL of 28 mM of sodium phosphate and 1 mL of 4 mM ammonium molybdate). The reaction mixtures were incubated at 95 °C for 90 min. The absorption of the mixture was measured at 695 nm in UV-Vis spectrometer (*UV-1601 PC*, *Shimadzu*, Kyoto, *Japan*). Positive control, ascorbic acid in the range of 100 – 1000 µg/mL was prepared and analysed in the same manner. The percent hydrogen peroxide scavenging activity which is the percentage inhibition was calculated using the formula below:

$$\left[\frac{(AB - AA)}{AB}\right] \times 100\ \%$$

Where, AB is absorbance of blank sample, AA is absorbance of crude extract (or positive control ascorbic acid). Calibration curve was obtained by plotting % inhibition against the concentration of the sample or positive control.

Peripheral blood mononuclear cells (PBMCs) isolation and culture

This study was approved by the University Research Ethics Committee of Universiti Brunei Darussalam (UBD/DVC/ 32.11). Peripheral Blood Mononuclear Cells (PBMCs) were isolated from heparinized blood samples from adult volunteers (n =3) by 1:1 dilution with RPMI 1640 and centrifuged at 400 x g over lymphoprep (Robin Scientific; 1.077g/l). Interfacial cells were recovered, washed and resuspended at

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10^6 cells/ml in Roswell Park Memorial Institute (RPMI) 1640 supplemented with 10% v/v Fetal Calf Serum (*FCS*), 100 U/mL penicillin/streptomycin, 10^{-2}M HEPES and 2 mM glutamine, referred to as 'complete medium', supplemented with 10 μg/ml phytohemagglutinin(PHA).

Lymphocyte Proliferation assay

Assays were performed in round-profile 96 well microtitre plates; 10^5 PBMCs was mixed with a range of extracts in RPMI 1640 complete media in a total volume of 200 μl and incubated at 37°C with 5 % CO_2 for up to 5 days. For every 1 ml of cell suspension, 1 μl of 5000 μMCellTrace™ Carboxyfluoresceinsuccinimidyl ester (CFSE) (Thermo Fisher Scientific, USA) was added to the suspension to achieve a 1:1000 dilution. The suspension was then incubated in a water bath maintained at 37°C for 20 minutes. Following incubation, 10 ml of RPMI 1640 was added to absorb the unbound CFSE stain and centrifuged at 350 x g for 5 minutes until a pellet was formed. The supernatant was decanted and the cell pellet was resuspended. The cells were washed twice in phosphate-buffered saline (*PBS*) before resuspension in the culture medium at 10^6 cells/ml.

Flow Cytometry

Cultured lymphoblasts were transferred to Fluorescence-activated cell sorting (FACS) tubes and spun down at 350 x g for 5 minutes to form individual pellets. The cells were re-suspended and washed twice in 2ml of PBS then resuspended in 200 μl of PBS. Propidium Iodide (PI) solution (5 μl; 0.5 μg/ml) was added to the cell suspensions and samples were immediately run through a BD Accuri™ C6 dual laser flow cytometer (BD Biosciences, San Jose, CA, USA). Data was analysed using BD Accuri C6 software and PI cell viability data was acquired from Fluorescence 3 (FL-3) plots. Modfit (v4.1.7) was used to evaluate cellular proliferation. Data was analysed by SPSS v21 and Prism (v7.05)[18].

Disc *Diffusion Assay*

The bacterial pathogens used were *Staphylococcus epidermidis, Vibrio* parahaemolyticus and *Pseudomonas aeruginosa* from the microbiology laboratory of Raja Isteri Pengiran Anak Saleha (RIPAS) Hospital. The exact amount of 100 μl of the respective bacterial culture (10^6 CFU/ml) was inoculated onto Mueller-Hinton agar and spread evenly. 5 μL of each *SF* extract was loaded onto sterile Whatman filter paper discs of 6 mm diameter. The final amountof extracts loaded on the discs were 0.5 mg, 1.0 mg and 2.5 mg of each *SF*extract dissolved in *DMSO.* The loaded discs were air-dried and then placed carefully onto respective sections of the agar. 5 μL *DMSO* was used as negative control while standard chloramphenicol or gentamicin (30 μg) was used as a positive control. The petri dishes were incubated for 18 hours at 37°C before the zones of inhibition of growth were measured. Three independent experiments were performed[22].

W*ell Diffusion Assay*

100 μl of the respective bacterial suspension (10^6 CFU/ml) was spread evenly onto Mueller-Hinton agar. Three different amounts of each extract were loaded into the 6 mm perforated well in the agar. The final concentrations of the extracts were 5 mg/well, 10 mg/well and 25 mg/well. Chloramphenicol or Gentamicin (50 μg/well) was used as a positive control and *DMSO* was used as negative control. The plates were allowed to stand for 30 minutes at room temperature prior to 18 hours incubation at 37°C. Zones of inhibition were measured to the nearest millimeter and three independent experiments were performed[23].

Results

Fractionation and characterizationof crude extract of SF

SF leaves were collected, cleaned and extracted after identification of the plant. The extraction was further subjected to fractionation and preliminary chemical

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characterisation.Column chromatography of the crude extract gave an initial separation of five fractions. Infrared spectroscopy and GC-MS were carried out for the crude extract and the obtained five fractions. Infrared spectra of the crude extract (Figure 2a) and the first fraction (Figure 2b) showed peaks characteristic of OH and C=O groups at 3343 cm^{-1} and 1635 cm^{-1} respectively. The carbonyl C=O peak was of much lower intensity in fractions 2-5 (Figure 2b), but still showed the presence of OH groups. The OH and C=O groups correlate well to structures of phenols, flavonols and flavonoids which also contain these functional groups.

Although a complete structure cannot be elucidated by GC-MS, an approximation of possible structures or fragments can be obtained. The mass spectrum of the crude extract is shown in (Figure 2c). The mass spectra at specific retention times (here 8.550, 14.942, 20.667 and 25.875 mins) were analysed and compared to an online database, as they were the largest intensity peaks that correlated to non-solvent peaks. GC-MS of the fractions (data not shown) gave similar plots to the methanol maceration crude extract which suggest that multiple fractionation steps or different solvent systems are required for the fractionation.

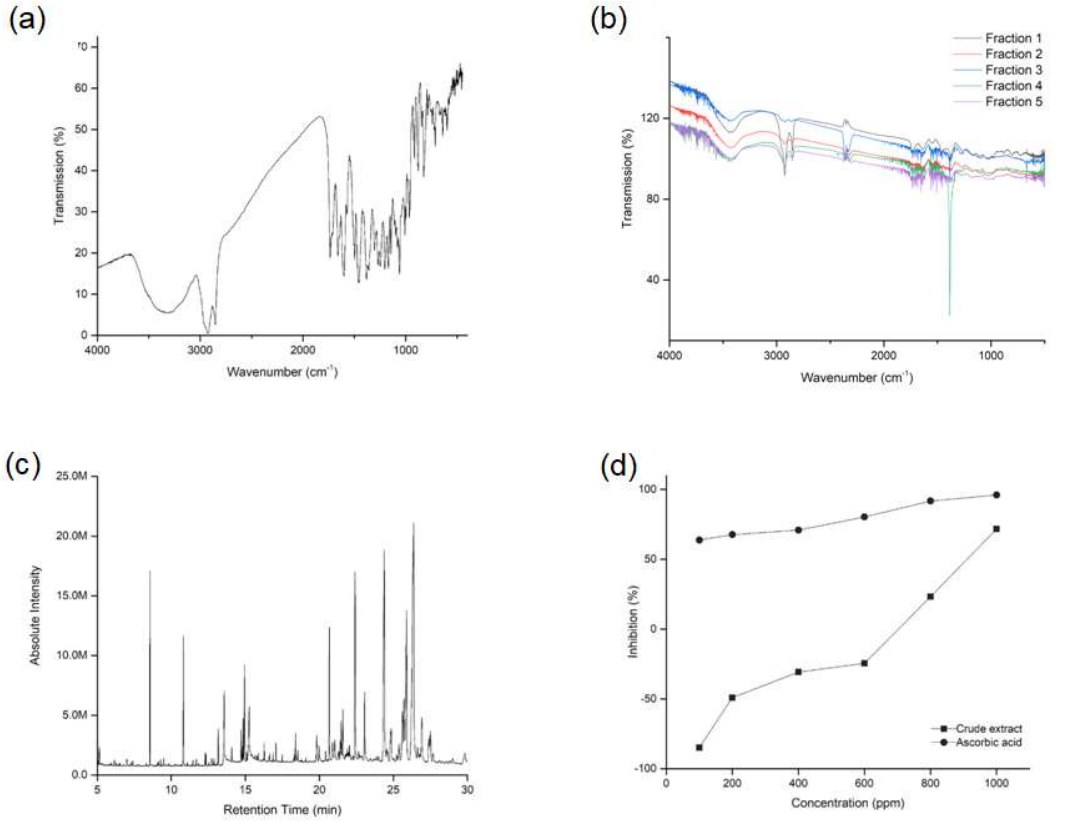

Figure 2: (a) Infrared spectrum of the crude methanol maceration extract. (b) Infrared spectrum of the fractions 1 – 5 obtained from column chromatography (c) *Gas chromatography with mass spectrometry (GC-MS)* of *Scurrula ferruginea* crude extract from methanol maceration. (d) Percent hydrogen peroxide scavenging activity (percentage inhibition) by the crude extract of *Scurrula ferruginea* of concentrations varying from 100 to 100 ppm

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In the analysis and comparison to the database, the focus was to search for compounds that contained phenols, phenyl ethers, and alkyl alcohols as these functional groups are the most likely to be found in the compounds from the plant. Compounds with functional groups similar to those we expect, with a similar mass spectrum fragmentation distribution to the database, and had the highest similarity in molecular mass with those from the GC-MS were picked out. From our findings, some of the compounds that were found to be in our extract were long chain alcohols, phytol (a diterpene alcohol and a constituent of chlorophyll), squalene (a triterpene and precursor to steroids, a component found in many plants), and lupeol (a triterpene which has been shown to have biological activities) [24–26]. The compounds can be separated by additional chromatography of the already separated fractions, for example using prep *TLC*, or *HPLC*. At a much later stage, characterization with *NMR* can be done to aid in the structural determination of the specific compounds.

Total *phenolic content and total antioxidant capacity of crude extract of SF*

The crude extracts were analysed for their total phenolic content and total antioxidant activity as the total antioxidant activity of many medicinal plants depends on the presence of polyphenols and flavonoids of plants. The total phenolic content is used to evaluate the quantity of phenolic compounds present in the crude extract which was found to be157.85 mg GAE/g. The potential of crude extract to reduce Phosphate-Molybdenum (VI) to Phosphate-Molybdenum (V) in thephosphomolybdenum assay showed an increase in percentage inhibition with increase in extracts concentration, which correlates to an increase in antioxidant activity (Figure 2d).

Antibacterial properties of SF extracts

The antibacterial activity of the leaf extracts of *SF*was evaluated in terms of zones of inhibition in disc diffusion assays against *Staphylococcus epidermidis*, *Vibrio parahaemolyticus* and *Pseudomonas aeruginosa.* For *Staphylococcus epidermidis,* the highest concentration at 2.5 mg/disc showed inhibition for all four extracts, while the two lower concentrations (0.5 mg/disc and 1 mg/disc) did not produce any zone of inhibition (Table 1). 2.5 mg/disc of aqueous extract presented the widest zone of inhibition (8.3 ± 0.0) mm against *Staphylococcus epidermidis* amongst the extracts.No inhibition was observed at 1 mg/disc or below from all the four extracts.As for *Vibrio parahaemolyticus,* the aqueous extract at 0.5 mg/disc showed a zone of inhibition (8.3 mm). Methanol macerated and methanol Soxhlet extracts of *SF*exhibited antibacterial activity at 2.5 mg against *Vibrio parahaemolyticus* with similar zone of inhibitions (9.0 mm each) for both the extracts. No zone of inhibition was observed against *Pseudomonas aeruginosa* with all four *SF* extracts at all tested concentrations (0.5 mg/disc, 1.0 mg/disc and 2.5 mg/disc) (Table 1).

The results obtained from disc diffusion tests were corroborated further with well diffusion assays. Table 2 illustrates the average zones of inhibition of *SF* extracts in a well-diffusion assay against *Staphylococcus epidermidis*, *Vibrio parahaemolyticus*at various concentrations. The three leaf extracts of *SF*(methanol macerated, aqueous maceration and methanol Soxhlet) extracts showed similar zones of inhibition at about 12 mmat concentrations of 5 mg/well against *Staphylococcus epidermidis* (Table 2). Increment in the extracts corresponds with increment in the zones of inhibition, illustrating the different rates of effectiveness of the extracts towards *Staphylococcus epidermidis.* Ethyl acetate extract gave the least inhibition amongst the extracts against *Staphylococcus epidermidis*.

Cytotoxicity *of SF extracts*

All extracts were cytotoxic to primary human PHA stimulated PBMCs (lymphoblasts) as evidenced by Propidium Iodide (PI) uptake.

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Table 1: illustrating the zones of inhibition exerted by various concentrations of *Scurrula ferruginea* discs

	Staphylococcus epidermidis			
Leaf extracts of *Scurrula ferruginea* (mg/disc)	Aqueous	Methanol Maceration	Ethyl Acetate	Methanol Soxhlet
0.5	0 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0
1	0 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0
2.5	8.3 ± 0.6	7.3 ± 0.6	7 ± 0.0	7.3 ± 0.6
30 µg/disc Chloramphenicol (positive control)	25 ± 0.0			
	Vibrio parahaemolyticus			
Leaf extracts of *Scurrula ferruginea* (mg/disc)	Aqueous	Methanol Maceration	Ethyl Acetate	Methanol Soxhlet
0.5	8.3 ± 1.0	0 ± 0.0	0 ± 0.0	0 ± 0.0
1	8.6 ± 1.0	0 ± 0.0	0 ± 0.0	0 ± 0.0
2.5	9 ± 0.6	9 ± 0.6	0 ± 0.0	8.3 ± 0.6
30 µg/disc Chloramphenicol (positive control)	18.4 ± 1.9			
	Pseudomonas aeruginosa			
Leaf extracts of *Scurrula ferruginea* (mg/disc)	Aqueous	Methanol Maceration	Ethyl Acetate	Methanol Soxhlet
0.5	0 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0
1	0 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0
2.5	0 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0
50 µg/disc Gentamicin (positive control)	13 ± 0.0			

Table 2: illustrating the zones of inhibition exerted by various concentrations of *Scurrula ferruginea* wells

	Staphylococcus epidermidis			
Leaf extracts of *Scurrula ferruginea* (mg/well)	Aqueous	Methanol Maceration	Ethyl Acetate	Methanol Soxhlet
5	12 ± 2.0	12.3 ± 0.6	0 ± 0.0	12 ± 0.0
10	13.7 ± 1.5	14.3 ± 0.0	8.3 ± 0.6	14 ± 0.0
25	16 ± 0.6	16 ± 1.0	10.3 ± 0.6	15.7 ± 0.6
50 µg/well Chloramphenicol (positive control)	25 ± 0.0			
	Vibrio parahaemolyticus			
Leaf extracts of *Scurrula ferruginea* (mg/well)	Aqueous	Methanol Maceration	Ethyl Acetate	Methanol Soxhlet
5	11 ± 1.0	8.3 ± 0.6	0 ± 0.0	0 ± 0.0
10	13 ± 1.0	8.6 ± 0.6	0 ± 0.0	8.3 ± 0.6
25	15.3 ± 0.6	14 ± 1.0	0 ± 0.0	14.6 ± 0.6
50 µg/well Chloramphenicol (positive control)	21.4 ± 1.4			

(*Contd.*)

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Table 2: illustrating the zones of inhibition exerted by various concentrations of *Scurrula ferruginea* wells (*Contd.*)				
	Pseudomonas aeruginosa			
Leaf extracts of *Scurrula ferruginea* (mg/well)	Aqueous	Methanol Maceration	Ethyl Acetate	Methanol Soxhlet
5	0 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0
10	0 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0
25	0 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0
100 µg/disc Gentamicin (positive control)	13 ± 0.0			

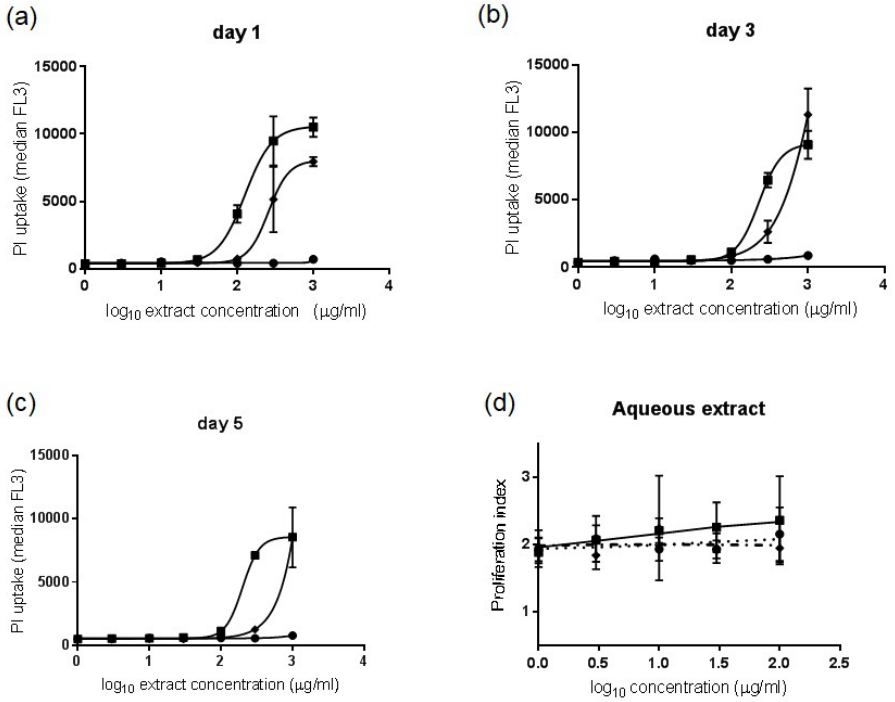

Figure 3: A representative dose response curve illustrating the effect of increasing concentrations of *Scurrula ferruginea* aqueous extract (circle), maceration extract (square), and Soxhlet extract (diamond) on phytohemagglutinin (PHA) stimulated PBMCs over time with (a) at day 1 (b) at day 2 (c) at day 5. Cell death is indicated by propidium iodide (PI) uptake by lymphoblasts. Data represent the mean of median FL3 values ±SD of triplicates. (d) A representative dose response curve of cell trace experiments indicating the effects of the aqueous extract of *Scurrula ferruginea* on lymphoblast proliferation over time (day 1 indicated by circles / dotted line; day 3 indicated by squares/solid line; day 5 indicated by diamonds/hashed line. Data represent the mean proliferation index ± SD of triplicates

This was observed in three different human samples. There was a significant increase in median fluorescence in the presence of each extract as low as 3 µg/ml ($p<0.05$; Figure 3). Solvent extracts of *SF* induced significant cell death, particularly at or above concentrations of 100 µg/ml. The aqueous extract caused less cell death than the methanol extracts

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(maceration and Soxhlet), but it was still evident.Cell trace experiments were performed in order to evaluate the effect of *SF* on lymphocyte proliferation, but the analyses assume there is no significant cell death. When the lower concentrations of the aqueous extract were incubated with human lymphoblasts, there was no significant effect on their proliferation ($p<0.05$; Figure 3).

Discussion

Both infrared spectrum and gas chromatography analyses have shown consistent contents of the extraction and fractionation. The characteristic functional group peaks observed in by infrared and gas chromatography mass spectrometry (GCMS) analyses corroborates each other. High intensity peaks of GCMS illustrated long chain alcohols squalenes, lupeols, and the other antioxidant substances which are usually found in the parasitic mistletoes [4,8]. Phosphomolybdenum assay indicated antioxidant activity by compounds extracted and fractionated from *SF* sample (Figure 2d) and interestingly, the compounds detected by both IR and GCMS have antioxidative properties.

The presence of antioxidative properties prompted the investigation of antibacterial properties of these extracts. Many plants, including *SF*, produce phenolic compounds to defend themselves against certain bacteria, exerting bacteriostatic or bactericidal effects [27,28]. In this study, the antibacterial results against the three bacteria (*Vibrio parahaemolyticus*, *Staphylococcus epidermidis* and *Pseudomonas aeruginosa)* yielded different results. From the data, extracts of *SF* showed antibacterial effects against *Staphylococcus epidermidis* and *Vibrio parahaemolyticus,* but not *Pseudomonas aeruginosa*. Data from both disc diffusion assay and the well assay correlate well against *Staphylococcus epidermidis*, indicating that all fractions inhibit *Staphylococcus epidermis,* with the ethyl acetate fraction having the least amount of zone of inhibition in both assays (Tables 1 and 2). The dipolar aprotic nature of ethyl acetate may have extracted less hydrophilic phytochemicals than the other solvents[29].

Vibrio parahaemolyticus, a pathogenic *Vibrios*strain, is a major causal organism of gastroenteritis and septicemia characterized by watery diarrhea, vomiting, fever and chills, sourcing from raw or undercooked, contaminated seafood such as raw oysters and mussels[30]. Table 1 illustrated that only *Vibrios parahaemolyticus* was susceptible to aqueous extracts of *SF* at 0.5 mg per disc. Congruent to the disc diffusion data for *Vibrios parahaemolyticus*, the largest zone of inhibition was found with the aqueous extracts of *SF*. Thus, the active compounds that act against *Vibrios parahaemolyticus* are of hydrophilic nature found within the aqueous extracts of *SF*. The strain of *Pseudomonas aeruginosa* used was obtained from a clinical setting. No inhibition of *Pseudomonas aeruginosa* by any extract of *SF*in either disc-diffusion assay or and well-diffusion assay. Thus, the compounds found within all the four *SF* extracts were not able to inhibit this bacterial strain, possibly due to either presence of suitable active compounds or adequate amount. This result differs from the Tunisian *Ruta chalepensis*, which inhibit *Pseudomonas aeruginosa* with diameter of inhibition of 17.7 mm,close to that of the standard antibiotic gentamycin[31]. A comparative analysis between the compounds of these two ethnopharmacological plants (*Ruta chalepensis*and *SF)* will shed light on the compounds that exert antibacterial effects against *Pseudomonas aeruginosa*.

Also, our study indicates that *SF* extracts are cytotoxic to primary human lymphoblasts in a time- and dose-dependent manner at concentrations between 3 μg/ml and 1000 μg/ml. This is in line with the findings of Marvibaigi et al [32]. Solvent extracts were more cytotoxic than aqueous extract of *SF*. Primary human lymphocytes was able to proliferate normally in the presence of lower doses of the aqueous extract of *SF* (3 μg/ml - 100μg/ml) with no apparent detrimental effects. This indicates

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the possibility of using *SF* extracts against a few cancer types.

However, there were a few limitations to our study. First, phytochemical screening of active constituents of plant such as flavonoids, alkaloids, tannins and phenols is required to authenticate our findings. Isolation and identification of these potential antibacterial compounds using methods such as Thin Layer Chromatography (TLC) and High-Pressure Liquid Chromatography (*HPLC*) will be useful for future work. Comparison of the mass spectrum at specific retention times with the mass spectra of known compounds from an online database resulted in an approximation of plausible compounds within the extract only. This study infers through the preliminary chemical characterisation and corroborated by various researchers, the presence of compounds including long chain alcohols, phytol (a diterpene alcohol and a constituent of chlorophyll), squalene (a triterpene and precursor to steroids, a component found in many plants), and lupeol (a triterpene which has been shown to have biological activities) from *SF* extracts. Further analysis with characterisation would be needed to confirm the identity of these compounds, and their specific biological functions [24,25,33]. Secondly, there is a lack of literature on the chemical profiles and GC-MS data on extracts of *SF*, A significant limitation of our study is the inability to pinpoint the specific molecules in the extracts of *SF*. The retention times mentioned were based on the relative higher intensity detected. The molecules postulated were based on mass spectrometry database and there may be other molecules of the same sizes, isomers of long chain molecules (alcohols) or metabolites not mentioned as most plants would have common secondary metabolites. Identification of the compounds responsible for different biological activities and the mechanism of action will be beneficial. Thirdly, the utilisation ofdisc diffusion and well diffusion assays for antibacterial work means that the minimum inhibition concentration of the potent extract cannot be determined. Therefore, future work to determine the minimum inhibition extract of the aqueous and methanol macerated extract of *SF* against both *Staphylococcus epidermidis* and *Vibrio parahaemolyticus* and broth dilution tests (such as the tube-dilution method to generate quantitative results) will shed better insights of the antibacterial effects of the extracts. In addition, these *in vitro* assays are unable to demonstrate any side-effects when these extracts are metabolized physiologically. Therefore, animal models are needed to investigate the therapeutic effects of the *SF*as well toxicity studies. Also, in most studies involving *SF*, the host plant of the mistletoe was not mentioned. This should be noted as few studies have shown that mistletoe growing on different host plant may have different chemical composition. A study of the chemical composition of the European mistletoe, *Viscumalbum* on three host plants has shown significant differences in their chemical composition which may, result in variation in their cytotoxic or antibacterial properties [34].

Therefore, the anticancer effects of *SF* remain a broad area of research to be done. Here, we have explored the phytochemistry, antioxidant, antimicrobial and cytotoxic potential of *SF* extracts. Although our results are still preliminary, we demonstrated that *SF* is a hemiparasitic plant harboring antioxidants with antimicrobial effects towards *Vibrios parahaemolyticus* and *Staphylococcus epidermidis*and cytotoxic effects towards PBMCs.

Conclusion

This study evaluates the properties of this interesting medicinal herb and highlights the potential anti-bacterial properties of *SF*. This work illustrates the selective antibacterial effects against *Staphylococcus epidermidis* and *Vibrio parahaemolyticus,* but not on*Pseudomonas aeruginosa.* In addition, this work sheds light on the antioxidative properties of various *SF* extracts and consequently, portrays the importance of future work on the potential toxicity of *SF*.

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Competing interests

The authors declare that they have no competing interests.

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Molecular Dynamics Study of Quinazoline Compounds Complexed with Filamenting Temperature-Sensitive Z Protein and Gyrase Subunit B as Potential Antibacterials

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Abstract

As the years go by, bacteria such as *Staphylococcus aureus* and *Mycobacterium tuberculosis* have developed resistance towards the current antibiotics which leads to ineffectiveness of antibacterial agents to kill or inhibit the bacteria. Thus, in order to overcome this issue, quinazoline derivatives have been proposed as the potential new antibacterial agents due to their antibacterial properties. Molecular dynamics, a computational technique, has been conducted in this study to determine the potential of quinazoline compounds as a novel antibacterial agent for *Staphylococcus aureus*'s DNA gyrase subunit B (GyrB) and *Mycobacterium tuberculosis*'s Filamenting temperature-sensitive Z (FtsZ) protein. Molecular dynamics simulation of the top 2 docked ligands of quinazoline for each FtsZ and GyrB were conducted by using Amber22 molecular dynamics simulation software. The analyses were conducted with *cpptraj*to evaluate the stability and binding interaction of the compounds with the target receptors.The dynamic studies of Q100 complexed with FtsZ show it is the most stable, with lower RMSD values (1.4Å for Q100 while FtzZ is 2.3Å) and lesser overall variation in RMSF. Although Q100 does not form a significant hydrogen bond with FtsZ, it has a higher negative free energy binding value (-25.48 kcal/mol) compared to Q56 with favourable hydrophobic and electrostatic interaction. While Q44 also shows the complex with GyrB is slightly more stable, with lower RMSF in residue 1 (3.0Å), stable RMSD (1.2Å for Q44 and 2.6Å for 3U2D), and a higher negative value of free binding energy (-23.21 kcal/mol) with favourable hydrophobic interaction. However, Q44 does not form a significant hydrogen bond as the occupancy is nearly zero. Q100 and Q44 have the most potential quinazoline derivative to act on the FtsZand GyrBrespectivelyto continue to the next step in drug design as a new antibacterial drug candidate.

Keywords: Molecular dynamics; Quinazoline derivatives; Filamenting Temperature-Sensitive Z Protein; DNA gyrase subunit B; AMBER

Introduction

As the years go by, bacteria have developed resistance towards the current antibiotics that were used in treating various types of bacterial infections through different mechanisms. The causes that enforce the bacteria to develop resistance against the current antibiotics has been reported is due to the excessive use and abuse of the antibiotics. In recent years, a large number of gram-negative and gram-positive bacteria have become unresponsive towards a wide range of antibiotics, and this leads to ineffectiveness of antibacterial agents to kill or inhibit the bacteria (1). Every year, more than 2.8 million antimicrobial-resistant infections occur and lead to more than 35,000 people die as a result in the United States based on the CDC's 2019 Antibiotic Resistance (AR) Threats Report. According

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to the World Health Organization (WHO), in 2019, at least 1.27 million of the people worldwide were killed due to antimicrobial resistance. The WHO thus mentioned antimicrobial resistance as an urgent global public health threat (2).

Heterocyclic compounds represent a vital group of biologically active compounds that may be useful in drug discovery and development. Compounds that consist of quinazoline, a nitrogen-containing heterocyclic, as its moiety has drawn the scientist's attention in the last few years because of their significant biological activities(3). Quinazoline possesses a wide range of pharmacological activities including anticancer, antifungal, anticonvulsant, analgesic, antibacterial etc. (3). Quinazoline structures bind at multiple sites with a high affinity due to its structure which benefits in medically active compound discovery to produce a novel drug to treat a particular disease(4). Thus, more future research on quinazoline structure can be done toprovide further insight in the pharmaceutical field.

Computer-Aided Drug Design (CADD) is a widely used technology that utilises computational methods to find, produce, and evaluate pharmaceuticals and other biologically active chemicals as well as tools for the modelling of compounds. By using CADD, researchers are able to study interactions between drugs and receptors with a lesser cost and effort in the pharmaceutical field in the drug discovery and development stage (5) thus allowing the development of new antibacterial to overcome the current situation of antibacterial resistance. CADD can be applied in molecular modelling which further aids in molecular docking as well as molecular dynamics (6).

Methodologies

The overview of this study is shown in Figure 1. The target structures which are

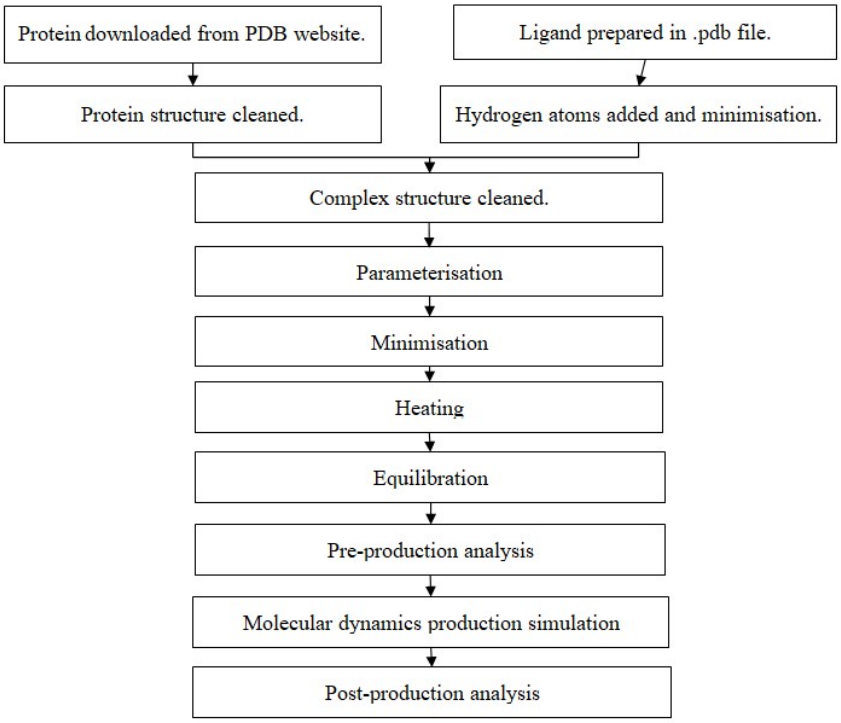

Figure 1: Overview of study

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FtsZ (PDB ID: 6Y1U) and bacterial GyrB (PDB ID: 3U2D) were downloaded from Protein Bank Data (PDB) website developed by Research Collaboratory Structural Bioinformatics (RCSB) and the non-amino acid residues such as water molecules and ligands which are present were removed. The top 2 ranked ligands for each target protein were extracted from molecular docking results (7) and hydrogen atoms were added into the ligand molecules. The files of both target protein structure and docked ligand obtained were merged in order to form the complex by using text editor. The files were then saved as ".pdb" format and were visualised to ensure complex structures were merged correctly by using Discovery Studio Visualizer (DSV). Molecular dynamics simulation was performed using the Amber22 and after preparing the topology and coordinate files in the software which ff19SB and GAFF force field was applied. The complex structures were solvated in the OPC water model explicitly and neutralised by the counterionssubsequently. Minimization was conducted and the complexes was heated up to 310K for a total of 30 ps while SHAKE algorithm is applied to constraint the hydrogen atoms with the cut off distance of 9Å. The complexes underwent equilibration steps with constant pressure for a total of 300 ps. Perl script was used in order to conduct pre-production analysis including the properties such as density, total energy, potential energy, kinetic energy, temperature, volume, and pressure to assess the stability of the complexes before further continuation to the production run. 5 ns of molecular dynamics production run was performed for each complex respectively and the trajectories were recorded for the final post-production analysis which includes the parameters such as H-bonds analysis, root mean square deviation (RMSD), root mean square fluctuation (RMSF), and Molecular Mechanics with Generalised Born and Surface Area Solvation (MM/GBSA) by using cpptraj module in the software used.

Results and Discussion

Pre-production Analysis

Results of the pre-production analysis which emphasis density, total energy, potential energy, kinetic energy, temperature, volume, and pressure for the top two quinazoline derivatives complexed with the respective target protein, DNA gyrase subunit B (3U2D) and FtsZ (6Y1U) are shown in Figure 2. The findings indicate that the quality of simulation isassured, and the complexes are stable throughout the simulation run evidenced by the equilibrated graphs presented in the figure (8).

Post-production Analysis

Root Mean Square Deviation (RMSD)

RMSD of the quinazoline derivatives complexed with the target proteins throughout 5 ns of production run are shown in Figure 3. The fluctuation of RMSD ranging from 0.5Å to 2.6Å for 3U2D receptor and 0.2Å to 1.2Å for Q44 compound indicates that the 3U2D-Q44 complex is stable as the values are within the range of 3.4Å (9). Meanwhile, the RMSD profile of another ligand, Q48, complexed with 3U2D shows that the fluctuation for the receptor is ranging from approximately 0.5Å to 2.5Å while the deviation of the Q48 compound is ranging from 0.1Å to 1.2Å. The deviations in the latter complex are also shown to be within the range (9), indicating that the 3U2D-Q48 are also stable throughout the MD simulation. In comparison, 3U2D-Q44 and 3U2D-Q48 complexes are stable and do not differ much in deviation of both ligand and receptor.

The RMSD of 6Y1U is within the range of 0.5Å to 2.6Å while Q100 compounds exhibit RMSD ranging from 0.2Å to 1.5Å. Since the results fall within the range of less than 3.4Å, the variation suggests that the 6Y1U-Q100 complex is stable (9). The findings for 6Y1U-Q56, indicate that the RMSD of the receptor is roughly between 0.5Å to 2.6Å, while ligand is ranging from 0.2Å to 2.3Å. Hence, 6Y1U-Q56 complex is considered stable throughout the

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5ns of MD simulations as the values show is within the acceptable range of less than 3.4Å (9) although significant deviation is observed in the RMSD of Q56. In contrast to the 6Y1U-Q56 complex, the 6Y1U-Q100 complex exhibits a slightly smaller variation of RMSD. Therefore, 6Y1U-Q100 is somewhat more stable than the other complex.

Root Mean Square Fluctuation (RMSF)

Based on the graph in Figure 4, the RMSF value at residue 1 in 3U2D-Q48 complex (4.1Å) is significantly higher than 3U2D-Q44 complex (3.0Å). Residue 184 in 3U2D-Q48 complex is also slightly higher with the value of 1.8Å while in 3U2D-Q44 is 1.2Å. Thus, residue 1 and residue 184 have a higher flexibility in 3U2D-Q48 complex during the simulation. Although residue 1 in both complexes has significantly higher peaks which suggest more flexibility than other residues in the complexes, only the RMSF values for 3U2D-Q44 are still within the permitted range of 3.4Å (9). Hence, Q44 is slightly more stable throughout the MD simulation than Q48 when binding to DNA gyrase subunit B.

The results of RMSF of the residues of the FtsZ is also demonstrated in Figure 4 which significant peaks are clearly shown in residue 56 in 6Y1U-Q56 and residue 161 in both 6Y1U-Q100 and 6Y1U-Q56 complex which suggests higher flexibility in these residues compared to the others. While the RMSF of most of the residues does not differ much in both complexes, 6Y1U-Q100 complex has shown more balanced peaks while 6Y1U-Q56 exhibits more significant fluctuations especially in residue 161 that reaches 2.5Å. However, the fluctuations in both complexes are still within the permitted range of 3.4Å (9). Hence, reveal that both complexes are stable throughout the MD simulations.

Binding Free Energy

Table 1 shows all quinazoline derivatives have negative binding free energy with the respective target proteins indicating

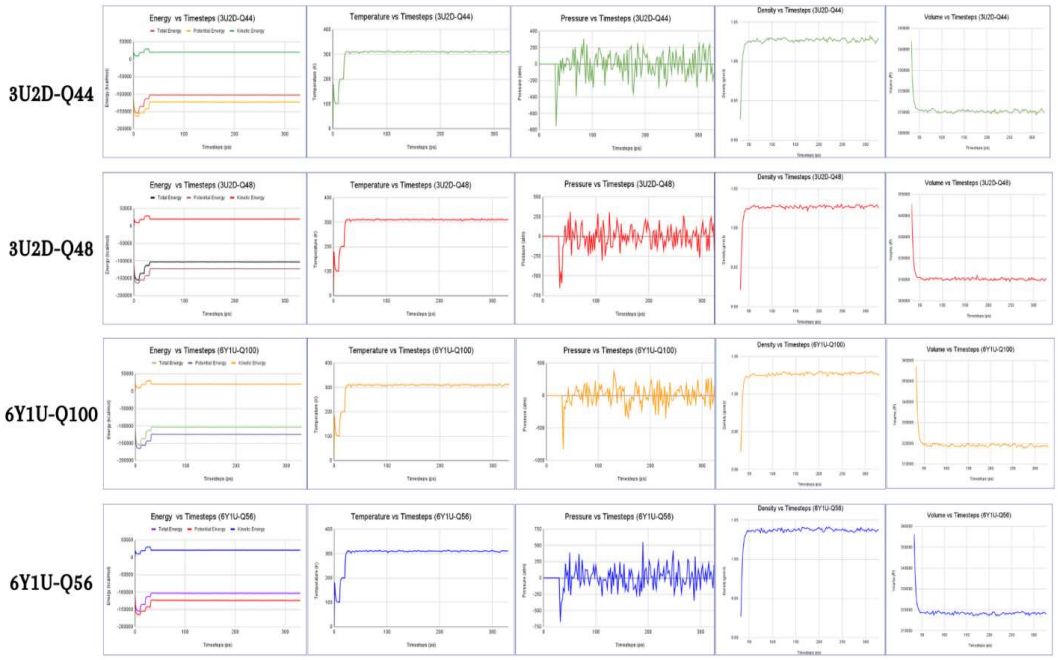

Figure 2: Pre-production analysis of quinazoline derivatives with the respective target proteins

Study of Quinazoline Compounds Complexed

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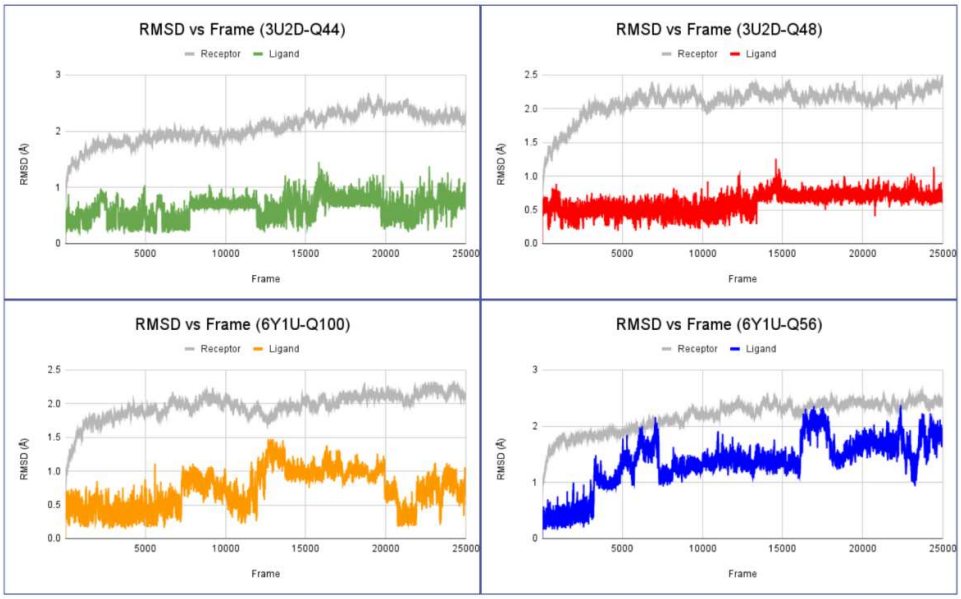

Figure 3: RMSD of the quinazoline derivatives complexed with the target proteins

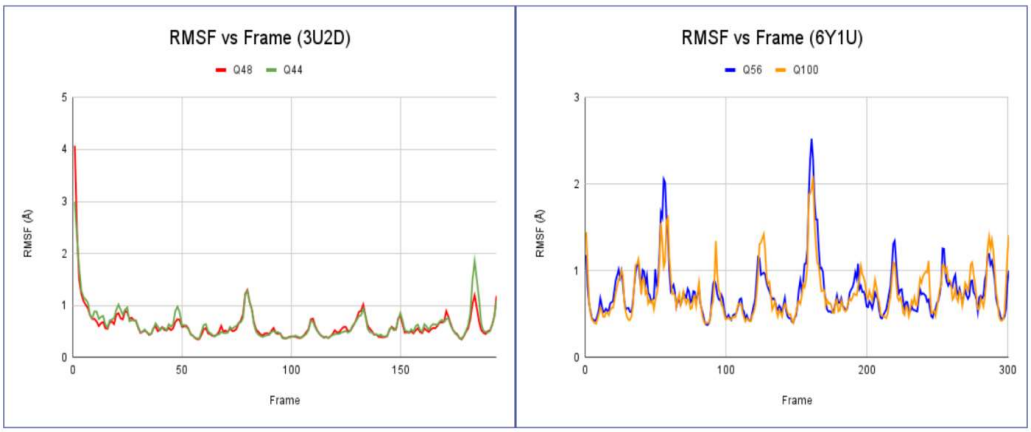

Figure 4: RMSF of the quinazoline derivatives complexed with the target proteins

that the binding of the compounds studied to 3U2D and 6Y1U respectively are favourable. Q44 with a slightly higher negative binding free energy of -23.21kcal/mol has a stronger binding to 3U2D receptor compared than Q48 with the binding free energy of -20.50 kcal/mol (10) which is mainly contributed by favourable Van der Waals energy in the system which is associated with hydrophobic interactions while the negative binding free energy for Q48 is contributed by favourable Van der Waals and electrostatic energy. The binding free energy findings for both compounds with 3U2D exhibit a good agreement with the docking findings (7) although the values are more negative than

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Table 1: MM/GBSA analysis of quinazoline derivatives complexed with target proteins

Quinazoline Derivatives Complexed with 3U2D						
Compound	Ebonded	EvdW	EEL	EGB	ESURF	ΔGbind
Q44	0.0027	-44.7601	1.5781	25.6118	-5.6407	-23.2082
Q48	0.0027	-46.3257	-3.2930	34.7930	-5.6800	-20.5031
Quinazoline Derivatives Complexed with 6Y1U						
Compound	Ebonded	EvdW	EEL	EGB	ESURF	ΔGbind
Q100	0.0008	-43.6219	-12.5674	36.0579	-5.3458	-25.4763
Q56	-0.0015	-34.1470	-18.5728	32.3359	-4.6551	-25.0406

Note: All values are given in kcal/mol. Ebonded: bonded energy; EvdW: Van der Waals Energy; EEL: Electrostatic energy; EGB: Polar solvation energy; ESURF: Non-polar solvation energy; ΔGbind: Binding free energy

molecular docking due to conformational changes in dynamics simulation.

Meanwhile, Q100 with a higher negative score, binds stronger to 6Y1U compared to Q56 (10). The highly negative binding free energy of Q100 and Q56 is mainly contributed by favourable electrostatic and Van der Waals energy in the system while the latter energy is associated with hydrophobic interactions. The binding free energy findings for both complexes exhibit a good agreement with the docking findings (7).

Binding Interaction

Q44 does not form significant hydrogen bonds as the occupancy of hydrogen bond form throughout the MD simulation, shown in Table 2, is less than 10% which should not be considered according to Nada et al. (2022). However, as shown in Table 1 previously, other binding interactions such as Van der Waals interaction (-44.76 kcal/mol) which is associated with hydrophobic interactions is present in the system but not electrostatic interaction due to positive value of the EEL energy (1.58 kcal/mol) which indicates the receptor and ligand slightly repels each other. Thus, in a nutshell, EEL is unfavourable while the hydrophobic interactions are strongly exhibited throughout the MD simulation of 3U2D-Q44 complex. These findings of the binding interactions specifically hydrogen bond interaction and electrostatic interaction do not demonstrate a good agreement with the docking findings (7) that mentioned hydrogen bonds should form in Ile51, Arg81, Arg84, Arg144 while Glu58 should form electrostatic interaction with Q44. This may be contributed by the ligand and protein dynamics and conformation changes over time of MD simulation (11). Meanwhile, significant hydrogen bonds are formed between O1 (11.2%) and O33 (31.6%) of Q48 with Arg144 as shown in Table 2. The presence of favourable electrostatic attraction and Van der Waals interaction are observed in previous Table 1 due to the negative value of EEL (-3.29 kcal/mol) and EvdW (-46.33 kcal/mol). In comparison with the results from docking study (7), partial agreement is

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Table 2: Occupancy of hydrogen bonds between quinazoline derivatives and 3U2D and 6Y1U

Quinazoline Derivatives Complexed with 3U2D			
Compound	Acceptor	Donor	Occupancy (%)
Q44	No significant occupancy		
Q48	LIG@O33	ARG_144@NH1	31.6
	LIG@O1	ARG_144@NH1	11.3
Quinazoline Derivatives Complexed with 6Y1U			
Compound	Acceptor	Donor	Occupancy (%)
Q100	No significant occupancy		
Q56	ASP_178@OD1	LIG@O2	44.5
	ASP_178@OD1	LIG@O1	32.0

Note: Only hydrogen bonds with occupancy >10% is displayed in this table

achieved in which hydrogen bond is formed in Arg144 except Arg84 with the ligand. Furthermore, presence of favourable electrostatic interaction and hydrophobic interactions can be observed in MD simulation which corresponds in the docking results for Q48 complexed with 3U2D.

Absence of significant hydrogen bond between Q100 and 6Y1U as the occupancy of between Q100 and 6Y1U are less than 10% which should not be considered (Nada et al., 2022) based on Table 2. Although no significant hydrogen bond is formed throughout the MD simulation in 6Y1U-Q100 system, favourable hydrophobic interaction (-43.62 kcal/mol) and electrostatic interaction (-12.57 kcal/mol) shown in Table 1 previously give rises to its favourable binding. Based on the intermolecular interaction result in the previous docking study (7), the hydrogen bond analysis in MD simulation demonstrates good agreement with the docking study that no conventional or carbon hydrogen is formed. The presence of favourable hydrophobic interactions can also be observed in both docking and dynamics study except electrostatic interactions where it is absent in the docking study. However, for Q56, specifically the O2 and O1, forms significant hydrogen bonds Asp178 of 6Y1U with the occupancy approximately 44.5% and 32% respectively as shown in (Figure 5).

In addition to hydrogen bond interaction, the findings in Table 1 show negative values of electrostatic energy (-18.57 kcal/mol) and VDW energy (-34.15 kcal/mol). Hence, indicates that favourable electrostatic and hydrophobic interactions are present within the 6Y1U-Q56 complex. This demonstrates a good correspondence with the docking study (7) specifically the presence of all hydrogen, electrostatic, and hydrophobic interactions although the number and type of hydrogen bonding residues are slightly different between docking study and dynamics. According to Zarezade V et al., 2021, the contribution of

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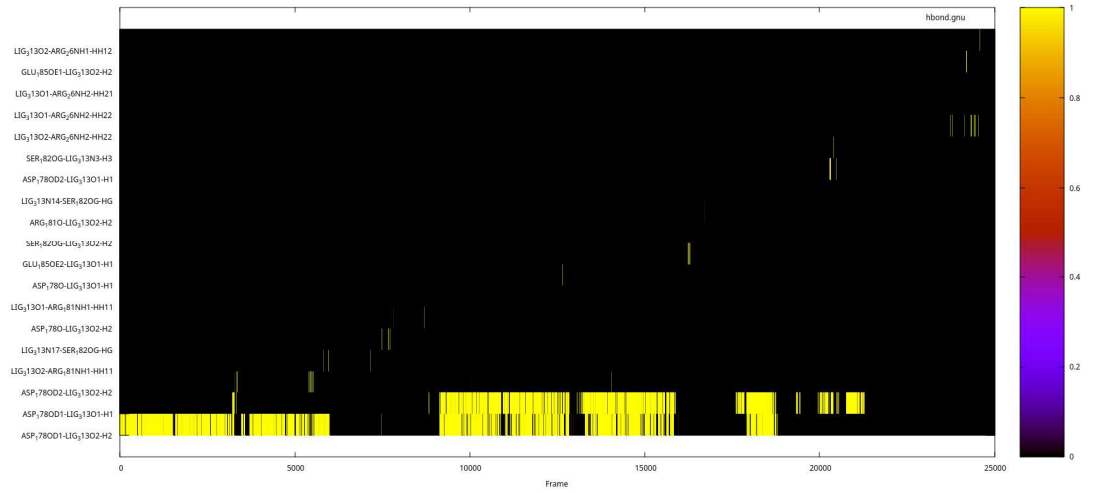

Figure 5: Hydrogen bond analysis of 6Y1U complexed with Q56

these different findings is due to conformational changes throughout the MD simulation.

Conclusion

Molecular dynamics simulation was conducted in this study to determine the potential of novel quinazoline derivatives as antibacterial agents by determining the stability, binding free energy, and binding interaction between the novel quinazoline derivatives and target proteins. All the compounds studied in this research have shown favourable binding free energy and inhibition constant in the docking study due to various intermolecular interactions between the quinazoline derivative compounds and target proteins. Compound Q44 demonstrates that the complex with DNA gyrase subunit B is slightly more stable than compound Q48, due to lesser fluctuation of tyrosine residue although both do not differ much in RMSD. Compound Q44 does not form significant hydrogen bonds or favourable electrostatics. However, its high negative value of vdW energy (-44.76kcal/mol) contributes to its favourable more negative binding free energy (-23.21kcal/mol). Meanwhile, Compound Q100 complexes with FtsZ show that it is more stable, with lower RMSD values in the ligand (1.5Å) while no significant difference in receptor compared to Compound Q56 (2.6Å). More balance peaks in RMSF are observed in Compound 100 and the higher negative free energy binding value (-25.48 kcal/mol) also contributes to its higher stability. However, no significant hydrogen bond is formed throughout the MD simulation. Thus, its low binding free energy is mostly contributed by hydrophobic interactions (-43.62 kcal/mol) and electrostatic interactions (-12.57 kcal/mol).

In summary, compound Q100 and Q44 have the most potential as the new drug candidates for its antibacterial properties and continue to the next step in drug design.

Contributions

MYY and IKM designed the experiments, MYY carried out the experiments, MYY has written the initial draft. MYY, and IKM, refined the manuscript. All authors have read and approved the manuscript.

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The cytotoxic and anti-depressant activity of ethanol extract of *Pterospermum semisaggitatum* (buch. – ham.exroxb.) leaves: an *in vitro* and *in vivo* study

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Abstract

This report investigates the phytochemical, cytotoxic and antidepressant property of the ethanolic extract of *Pterospermum semisagittatum* from South Asia region. Extracts subjected to pharmacological activity using *in vitro* and *in vivo* methods which are cytotoxic and antidepressants activity on animal model alongside its phytochemical profiling. The cytotoxic activity was investigated using brine shrimp lethality assay and antidepressant activity was determined by forced swimming test (FST) and tail suspension test (TST) in mice. The plant extract of ethanol extract of *Pterospermum semisagittatum* (EEPS) was found secondary metabolites, notably steroids, glycosides, tannins, flavonoids, saponins, and alkaloids etc. EEPS showed substantial cytotoxic effects lethal concentrations (LC_{50}) value are 347.65 µg/mL. Besides, treatment with EEPS revealed a significant reduction of immobility time in a dose-dependent manner in Force Swimming Test (FST) and Tail Suspension Test (TST). The dose of 400 mg/kg, body weight shows the best antidepressant effects in both FST and TST which are respectively immobile time 198.5± 3.81**** and 157.5 ± 4.01**** statistically significant (****P < 0.0001). Subsequently, the dose of 200 mg/kg, body weight shows the best antidepressant effects in both FST and TST which are respectively immobile time 180± 3.03**** and 33.75± 2.95**** (statistically significant).The investigation concludes that EEPS can be a potent source of antidepressant and cytotoxic agents.

Keywords: Ethanol; *Pterospermum semisagittatum*; phytochemicals; cytotoxic and antidepressant.

Introduction

The biological resources found in nature, such as plants, animals, and microbes, can be utilized for various purposes such as medicinal, social, and environmental goals. There are a vast number of medicinal plants around the world that exhibit diverse pharmacological effects. These medicinal herbs are a valuable gift from nature that can aid us in living a healthy and illness-free life. (Akanda & Hasan, 2021; Alam et al., 2021; Arman et al., 2022). In order to help people live a healthy, disease-free life, medicinal plants are nature's gift to people (Parves, 2016). For millennia, medicinal plants have been used, and their bioactive natural compounds play a significant role in safeguarding health in both the pharmaceutical and food industries, as well as contributing to the fragrance, agricultural, and personal-care product sectors (Rasool,2012;Guha et al., 2021). Approximately 70% of new pharmaceutical compounds and their derivatives are derived from plants (Du & Tang, 2014). Complementary and alternative medicine (CAM) encompasses a broad spectrum of diagnostic and therapeutic techniques in dermatology that complement conventional

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dermatological procedures, incorporating the latest scientific advancements and CAM practices. (Tirant et al., 2018). Around 80% of the global population uses traditional botanical remedies, according to the World Health Organization (WHO), with 40,000 to 70,000 species of medicinal plants used as traditional remedies across the globe(Hosseinzadeh et al., 2015; Verpoorte et al., 2006). Discovering lead compounds that possess the necessary biological activity is an essential part of creating a new medication. These lead compounds, which can have various beneficial effects such as fighting cancer, bacteria, reducing pain, relieving diarrhea, lowering blood sugar, and acting as antidepressants for the central nervous system, are already present in nature. (Islam et al., 2019). The evaluation of the crude plant extract for its purported medicinal benefits often occurs at the beginning of the investigation for the discovery of bioactive components (Chandrakant et al., 2012). In Malaysia, the plant grows abundantly and adds to the beauty of the parks and roadsides, making it an attractive sight. Throughout history, it has been believed that medicinal herbs are a vital source of sustenance for human beings. Utilizing plants and natural products derived from plants is essential for promoting good health due to their rich variety of nutritional benefits. These include vitamins, minerals, phenolic compounds, fiber, antioxidants, and bioactive metabolites, all crucial for maintaining well-being. (Khan et al., 2020).

Phytochemicals produced by plants are classified into two main categories, primary and secondary metabolites. Primary metabolism involves the creation and breakdown of essential substances such as proteins, lipids, nucleic acids, and carbohydrates, which are necessary for all living organisms. The substances involved in these metabolic pathways are known as primary metabolites(Velu et al., 2018). The production of secondary metabolites or natural products by an organism is a process that is often unique to the organism or is a reflection of the species' distinct characteristics. Unlike primary metabolites, secondary metabolites are not usually essential for an organism's growth, development, or reproduction. Instead, they are created as a result of the organism adapting to its environment or as a potential defense mechanism against predators, aiding in the organism's survival (Velu et al., 2018; Dhaniaputriet al., 2022). According to Harborne and Baxter (Harborne et at., 1993), the primary aim of qualitative phytochemical analysis is to provide a preliminary assessment of the presence or absence of certain classes of compounds in plant extracts such as alkaloids, flavonoids, saponin, tannin etc. These substances are recognized for their pivotal role in the medicinal and therapeutic attributes exhibited by plants. Therefore, the identification of these bioactive compounds is necessary to ensure the efficacy and safety of medicinal plant products.

Cytotoxicity tests are important methods for evaluating the potential toxicity of compounds and extracts from medicinal plants. These tests are commonly used in drug discovery and development to identify compounds with potential anticancer and antiproliferative activity. One common cytotoxicity assay is the brine shrimp lethality assay, which has been used for preliminary screening of potential anticancer and antiproliferative agents due to its simplicity, cost-effectiveness, and ability to test large numbers of samples in a short period of time (Mosmann,1983). Additional frequently employed cytotoxicity assessments comprise the MTT assay, gauging cellular mitochondrial activity, and the lactate dehydrogenase (LDH) assay, quantifying the discharge of lactate dehydrogenase stemming from cell injury. These assays are more advanced and are often used to confirm the results of brine shrimp lethality assays (Van Meerloo et al., 2011).

Neurodegenerative diseases are a set of long-term and progressive ailments that impact the central nervous system, causing deterioration in cognitive ability, motor

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functions, and behavior. The incidence of neurodegenerative diseases is on the rise worldwide, with estimates suggesting that the number of people affected by these disorders will triple by 2050 (Pringsheim et al., 2014). Neurodegenerative diseases are complex and progressive disorders affecting the central nervous system, resulting in cognitive and motor function decline. Polyphenols, alkaloids, terpenoids, and flavonoids are among the phytochemicals with neuroprotective effects by modulating pathways related to oxidative stress, inflammation, and protein aggregation (Shallie, 2020). Antidepressants are pharmaceuticals employed in the management of diverse mental health issues like depression syndromes, anxiety like disorders, and OCD(Obsessive-Compulsive-Disorder). They function by modifying the concentrations of neurotransmitters within the brain, which play a pivotal role in regulating mood, behavior, and emotions. Tricyclic antidepressants (TCA), monoamine oxidase inhibitors (MAO inhibitors), selective serotonin reuptake inhibitors (SSRI), serotonin antagonist and reuptake inhibitors, norepinephrine and dopamine reuptake inhibitors, and serotonin and norepinephrine reuptake inhibitors are all indispensable players in the diverse pharmacological arsenal deployed to combat depressive disorders (Arroll et al., 2016). The flora for example *Pterospermum semisagittatum* is naturally occurring in Asia. This plant is typically employed as complementary therapies to treat ailments including malignancies, tumors, cardiac palpitations, sensations of burning, hepatic ailments digestive problems, respiratory tract disorders, skin disorders, malaria, as well as rheumatic pain (Taraquzzaman et al., 2014). The aim of this investigation was to delve into the ingenious crafting of groundbreaking bioactive compounds derived from the wondrous *Pterospermum semisagittatum*. These compounds were tailored with the captivating goal of significantly mitigating toxicity while fostering remarkable recuperation from various disorders.

Materials and methods

Drugs and chemicals

The substances used in the research included ethanol (R & M Chemicals, Selangor, Malaysia). Fluoxetine (Eskayef Banlgadesh Ltd, Bangladesh) and the remaining chemicals were purchased from a local vendor through Chemiz Ltd in Malaysia.

Collection, identification of plant and extraction

Fresh *Pterospermum semisagittatum* leaves were collectedlocally from the Putrajaya, Malaysia. The plants were subsequently identified by Dr. Khairil Mahmud, the Biodiversity unit at the Institute of Bioscience at the University of Putra Malaysia, as well as Dr. Nor Azam Bahari, the attending veterinarian at the same institution. After being shade-dried, the dried leaves were then crushed into the form of powder by means of a mechanical crusher (Sieve No. 10/44). Subsequently, the powder had been extracted for seven consecutive days in a row by using laboratory-grade ethanol and a Soxhlet apparatus (Dhaniaputri et al., 2022). Concentrated crude ethanol extract (EEPS) was prepared by evaporating the resulting extracts by means of a Rotavapor (Buchi Flawil, Switzerland) at reduced pressure. The extract had been preserved cooled till it was required for the purpose of the investigation.

Experimental design

To perform the in vivo study, twelve mice were separated into four groups (Group I-IV), with each group consisting of three animals (n = 3). The therapy procedure was developed as follows: Group received the vehicle (1% tween 80 in distilled water, 10 mL/kg b.w., p.o.), Group received the standard medicine (Fluoxetine HCl 20 mg/kg b.w., p.o.), and Groups and IV received EEPS 200 and 400 mg/kg b.w., p.o. respectively.

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Qualitative phytochemicals study

The qualitative phytochemical study was analyzed using standard protocols (Khan et al., 2020) which involved checking for the presence of secondary metabolites such as flavonoids, alkaloids, carbohydrates, protein & amino acid, saponins, glycosides, steroids, tannins, phenols, cholesterols, resins and reducing sugar (Table 1).

Cytotoxic assay by brine shrimp lethality bioassay

To determine cytotoxic action, crude extracts of *P. semisagittatum* leaves were tested using a brine shrimp mortality bioassay. The process commenced with the careful incubation of Artemia salina, more commonly known as brine shrimp eggs, within a specialized water reservoir enriched with sea salt. Within a cozy environment maintained at a temperature range of 22-29°C, the tiny nauplii emerged, drawn towards a radiant light source positioned strategically on one side of the vessel. These nascent organisms were then delicately harvested and injected into miniature buckets brimming with pristine seawater, a process repeated 2-3 times to ensure optimal cultivation. The test dosage was gradually increased to 800, 400, 200, 100, 50, 25, 12.5 and 6.25 µg/ml. Vincristine and DMSO were used as positive and negative controls, respectively, to compare to the test group (Pisutthanan et al., 2004;Rahman et al., 2013). This finding was used to determine the percentage of mortality in brine shrimp nauplii at different saturation level.

Forced swimming test (FST)

The FST method proved efficacious in evaluating the antidepressant impact on

Table 1: Phytochemical screening of ethanol extract of *P. semisagittatum* leaves

Sl.	Phytochemicals	Name of the tests	Observation
1	Flavonoids	Wagner's reagent test	+
2	Alkaloids	Wagner's reagent test	+
3	Carbohydrates	Benedict test	+
4	Protein & amino acid	Sulphuric acid test	+
5	Saponins	Foam height test	-
6	Glycosides	Keller-killiani test	+
7	Steroids	Salkowski test	-
8	Tannins	Lead acetate test	+
9	Phenols	Iodine test	+
10	Cholesterols	Salkowski test	+
11	Resins		-
12	Reducing sugar	Benedict test	+

(+) means present, (-) means absent

Values have been presented as mean ± SEM (n = 3) with the P value ($^{****}P < 0.0001$) being statistically significant in comparison to the control group ($P > 0.05$) processed by Dennett's test by using one-way ANOVA analysis (graph pad prism software, version 8.4.4) for multiple comparisons. EEPS = Ethanolic extract of *Pterospermum semisagittatum*

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Swiss albino mice. According to the requirements of the research design, the therapy was delivered a half-hour before to the experiment. The experimental mice had been kept individually in an open cylindrical box with a 19 cm depth of water at a constant temperature of 25 °C (10 cm height x25cm diameter). After using each mouse to execute an FST, the water within the compartment changed since "used water" was utilized to change the games. Within the first two minutes of the test, every mouse displayed complete mobility at some point. At some point during the following four minutes of the total six minutes of checking out time, the period of inactivity was manually recorded. Mice were deemed immobile if they remained afloat without exhibiting any movement beyond what was essential to maintain their heads above the water. Mice in each of the three groups received the same care (Islam et al., 2021; Khan et al., 2020).

Tail Suspension Test (TST)

To assess the behavioral effects of antidepressants on mice, the TST was employed. According to the research strategy, the therapy was administered 60 minutes prior to the start of the experiment. In order to determine the total length of immobility brought on by tail suspension, mice were suspended 50 cm above the ground, positioned on the table's edge, with adhesive tape securely affixed just 1 cm from the tip of each mouse's tail.. Each mouse from all agencies was timed over the final four minutes of a six-minute period. An animal hanging passively and without motion ceased to exhibit any frame motion when it was measured to be motionless. Mice in each group received the same care (Hossen et al., 2021; Vickers, 2017).

Statistical analysis

The values were showed in mean ± standard error mean (SEM). $P < 0.05$statistically significant, which was carried by one-way ANOVA (Dunnett's test) using GraphPad Prism (version 8.4.) software.

Results

Qualitative phytochemicals study

The qualitative analysis of phytochemicals in this study indicated the presence of flavonoids, alkaloids, carbohydrates, proteins & amino acids, glycosides, tannins, phenols, cholesterols, and reducing sugars.

Cytotoxic assay by brine shrimp lethality bioassay

Brine shrimp lethality activity of the ethanolic extracts of *P. semisagittatum*was presented in (Figure 1). The crude extract showed 75% mortality at 800 µg/mL concentration and LC_{50} value was 347.65µg/mL which was were considered significantly active. No mortality was found in negative control (DMSO) group.

Effects of EEPS on FST and TST

Figures 2 and 3 illustrate the effects of oral administration of the EEPS solution on immobility time in the FST and TST respectively. As depicted in figures, the extract was given by oral route at doses of 200 and 400 mg/kg significantly ($^{****}P < 0.0001$) decreased the immobility time in both TST whereas 200 and 400 mg/kg revealed significant ($^{****}P < 0.0001$ and $^{***}P < 0.001$) decrease in a dose-dependent manner as compared to the control group. In order to rule out the possibility that the extract's anti-

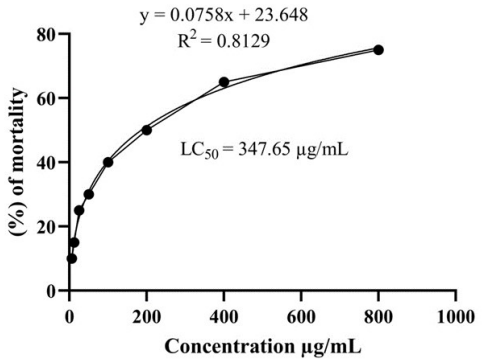

Figure 1: Cytotoxicity activity of EEPS

The cytotoxic and anti-depressant activity of ethanol extract

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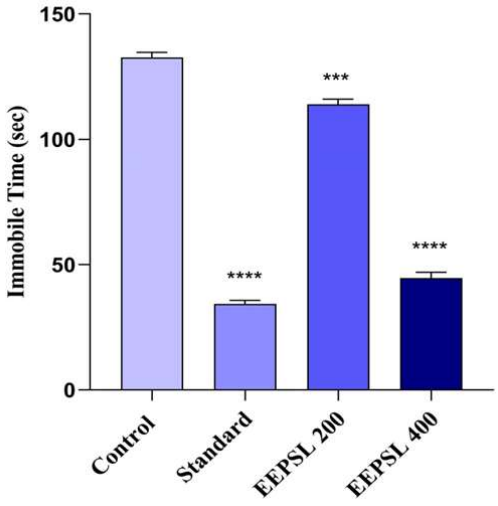

Figure 2: Antidepressant activity of EEPS through Forced swim test (FST) test in *Swiss albino* mice

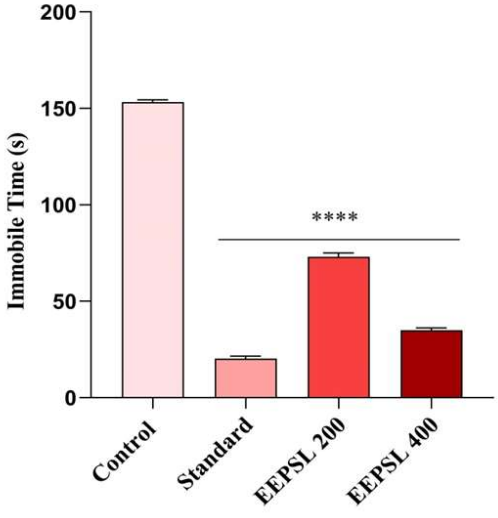

Figure 3: Antidepressant activity of EEPS through Forced swim test (TST) test in *Swiss albino* mice

immobility effect was attributable to a psychostimulant impact, the dosages that generated an effect in the FST were given to a separate group of mice who were then evaluated in the TST.

Discussion

Phytochemical screening is an important element of plant-based research since it detects the existence of various chemical substances that may have therapeutic advantages. The EEPS was subjected to a qualitative phytochemical study, which revealed varied quantities of carbohydrates, alkaloids, steroids, glycosides, tannins, flavonoids, saponins, terpenoids, and phenols. The plant's biological effects might vary depending on the phytochemicals present (Khyade et al., 2014).

Several studies have demonstrated the usefulness of the Brine Shrimp Lethality Assay(BSLA) in screening for the toxicity of different compounds. For example, researchers have used this assay to evaluate the toxicity of plant extracts (2), marine natural products (3), and various pharmaceuticals (4) (McLaughlinet et al., 1998). One advantage of the BSLA is that it can be used to screen a large number of compounds rapidly and inexpensively. Moreover, the BSLA is a relatively simple assay that requires minimal equipment and expertise. However, it is important to note that the BSLA is a preliminary screening tool and that further testing, such as in vivo studies, is necessary to confirm the toxicity of the compounds identified in this assay. By plotting the proportion of shrimp deaths against the logarithm of the sample concentration (toxicant concentration), the lethal concentration (LC_{50}) of the test samples was determined after 24 hours. The best-fit line was then determined from the curve data using regression analysis.

Forced Swim test involves placing a mouse in a container of water from which it cannot escape, and measuring the time the mouse spends immobile or struggling to escape over a defined period of time. Several studies have used the FST to evaluate the antidepressant activity of various drugs, including selective serotonin reuptake

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inhibitors (SSRIs), tricyclic antidepressants (TCAs), and monoamine oxidase inhibitors (MAOIs). For example, a study by *Porsolt et al*. (Porsolt et al., 1997) showed that acute treatment with the SSRI fluoxetine reduced immobility time in mice, indicating antidepressant activity.However, there are also criticisms of the FST as a measure of antidepressant activity. Some researchers argue that the test is highly influenced by non-specific factors such as stress and fatigue, and that it does not necessarily reflect the clinical efficacy of antidepressants in humans (Cryan et al., 2005). High rates of chronicity, recurrence, and suicide are all related with the serious but common condition of depression. Although there are numerous antidepressant medications available, their use is sometimes accompanied by unpleasant side effects, and some depression patients have a limited response to their therapeutic benefits (Kwon et al., 2010). The EEPS demonstrated a clear antidepressant-like effect in both the forced swimming and tail suspension tests in the current study. The immobility of mice occurs in a condition of despair or reduced mood, which is similar to human depression (Foyet et al., 2011). The mice had given up on the idea of escaping the restricted region. It has been shown that antidepressant medications can shorten the length of immobility in the animal model (Abelaira et al., 2013). Antidepressants activity of plant extracts may be due to the presents of alkaloid, carbohydrate, flavonoid and saponin (Bahramsoltani et al., 2015) and the presents of alkaloids, carbohydrates, flavonoids and saponin represents the function of EEPS.

Conclusion

Finally, the study underscores *Pterospermum semisagittatum's* potential as a source of bioactive chemicals having pharmacological properties, such as cytotoxicity and antidepressant effects. The presence of phenolic and flavonol components in the ethanolic extract of *P. semisagittatum* indicates that these phytochemicals may be responsible for the observed bioactivity. The findings of this study provide a foundation for further research into the active compounds' mechanisms of action and potential as lead molecules for medication development. Natural products, such as *P. semisagittatum,* can be used to generate novel medications that are safer and more effective than traditional pharmaceuticals.

Contributions

MHU and IKM designed the experiments, MHU carried out the experiments, MHU has written the initial draft. MHU and IKM, refined the manuscript. All authors have read and approved the manuscript.

Ethics approval

Cyberjaya University Animal Care & Use Committee (CACUC), wide reference number CACUC/1/2023/3.

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Phyla Nodiflora Derived Silver Nanoparticles In Hydrogel Formulations: A New Approach To Wound Management

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Abstract

A vital component of healthcare is wound healing, which includes the healing of both acute and chronic wounds. Prolonged inflammation brought on by immunological dysregulation, increased reactive oxygen species, and matrix metalloproteinase activity make chronic wounds difficult to heal. Recent developments in hydrogel dressings supplemented with antioxidants and immunomodulatory medicines have the potential to improve chronic wound healing. This work investigates the synthesis of green-synthesized silver nanoparticles (AgNPs) from the medicinal plant *Phyla nodiflora* , which has a variety of pharmacological properties, intending to develop a novel phytopharmaceutical hydrogel. Ethanolic extraction was employed to obtain bioactive compounds from *Phyla nodiflora* , followed by comprehensive phytochemical analysis revealing a notable amount of alkaloids, flavonoids, tannins, and phenolic compounds. The green synthesis of AgNPs has been examined by UV spectroscopy, zeta potential measurement, SEM with EDAX, and HRTEM. The hydrogels were evaluated for their pH, viscosity, homogeneity, spreadability, drug content, and release profiles. The optimized formulation demonstrated exceptional spreadability and prolonged medication release, making it appropriate for the treatment of chronic wounds. The hydrogel's biocompatibility and wound-healing capabilities were shown in *In-vitro* experiments, suggesting that it may find use in clinical settings for the treatment of chronic wounds in the future.

Keywords: Wound Healing, Chronic Wounds, Silver Nanoparticles, *Phyla nodiflora*, Green Synthesis, Phytopharmaceutical Hydrogel

Introduction

A vital component of healthcare is wound healing, which includes the restoration of both acute and chronic wounds. Due to immunological dysregulation, pro-inflammatory macrophages, increased reactive oxygen species, and higher matrix metalloproteinase activity, chronic wounds have extended inflammation and poor healing. Chronic wound healing is hampered by biological biofilms. Recent developments in the study of wound healing have focused on immunomodulatory treatment approaches, including altering the phenotypes of macrophages, controlling the expression of miRNA, and employing both pro- and anti-inflammatory medications. Antioxidant-infused hydrogel dressings show promise for hastening wound healing and laying the

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groundwork for upcoming advancements in the treatment of chronic wounds(1).

The skin contains several subsets of stem cells that have multipotent qualities when it is wounded, therefore understanding the roles of different cell types in wound healing is essential. The functional and phenotypic diversity among these cell types has been revealed by advances in single-cell technologies, and this knowledge can inform the development of therapeutics targeted at improving tissue damage and wound healing. The study of the clinical and molecular foundations of wound rehabilitation is crucial because chronic, non-healing wounds, which are frequently linked to aging and illnesses like diabetes, entail substantial financial costs(2).

Native to southern India, *Phyla nodiflora* is a medicinal plant with a wide range of pharmacological activity, such as hypotensive, anti-inflammatory, antioxidant, anti-tumor, antibacterial, anti-diarrheal, anticancer, and diuretic effects(3–5). It is used in traditional medicine to treat women in state, diarrhea, dysuria, and digestive issues. It is eaten in Ceylon, but it is sipped like tea in the Philippines.In Pakistan's tribal people, the herb is also utilized as a traditional cosmetic and cure for many skin ailments(6).

The green synthesis of silver nanoparticles (AgNPs) has garnered attention for their broad-spectrum antibacterial properties, making them suitable for biomedical applications such as wound care. This environmentally friendly synthesis method employs plant extracts, reducing harmful chemical pollutants. The green synthesis process rapidly produces metal nanoparticles under mild conditions, leveraging phytochemicals like terpenoids, aldehydes, alkaloids, amino acids, ketones, flavonoids, and carboxylic acids. This method is cost-effective, environmentally sustainable, and avoids generating hazardous by products(7,8).

Hydrogels, known for promoting epithelial renewal and maintaining a moist wound environment, show significant potential in wound dressing applications(9). This study aims to develop a novel phytopharmaceutical hydrogel for wound healing, utilizing *Phyla nodiflora* -derived silver nanoparticles synthesized through green methods. Using an ethanolic extract of *Phyla nodiflora* silver nanoparticles was synthesized and incorporated into a carbopol hydrogel formulation. Various formulation trials were conducted, with the optimal formulation selected based on comprehensive evaluation parameters.

Materials and Methods

Extraction and evaluation of the plant

Preparation of Ethanolic Extract of *Phyla nodiflora*

The plant was collected from an herbal garden in Chennai, Tamil Nadu, and authenticated by a botanist. The entire *Phyla nodiflora* plant was air-dried and subsequently ground into a coarse powder. Three kilograms of this dried material were subjected to maceration with ethanol for seven days. Following maceration, the extract was filtered and concentrated using a rotary evaporator under reduced pressure and low temperature. The yield of the ethanolic extract was then calculated and documented.

Preliminary Phytochemical Screening

The ethanolic extract of the plant subjected to preliminary phytochemical screening to identify the presence of alkaloids, proteins, saponins, starch, amino acids, steroids, terpenoids, tannins, flavonoids, anthraquinones, and glycosides using conventional qualitative methods(10).

Quantitative Analysis

The quantitative analysis of the ethanolic extract of *Phyla nodiflora* was conducted to determine the precise concentrations of these phytochemicals.

Estimation of Total Phenol Content- Folin Ciocalteu method

Using gallic acid as a standard, the Folin-Ciocalteu technique was used to calculate the total phenol concentration.

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Making a calibration curve from standard gallic acid solutions at 200 mg/mL, 300 mg/mL, 400 mg/mL, and 500 mg/mL was the process. To perform the experiment, combine 1 mL of Folin reagent, 5 mL of distilled water, and 1 mL of the sample. One mL of 10% sodium carbonate was added after five minutes, and the mixture was allowed to stand at room temperature for an hour. At 725 nm, absorbance was measured. Gallic acid equivalent (GAE) in milligrams per gram of dry extract weight was used to express the results.

Estimation of Tannin Content- Folin-Denis method

The total tannin content was measured using the Folin-Denis technique. The extract (1 mg/mL) was produced as a stock solution. 0.5 mL of the Folin-Denis reagent, 0.5 mL of distilled water, and 0.1 mL of the extract were combined for the experiment. After adding 1 mL of 15% sodium carbonate to the mixture, it was allowed to stand at room temperature in the dark for half an hour. At 700 nm, absorbance was measured. The standard used was 1 mg/mL of tannic acid, and the data were displayed on an estimation graph(11).

Estimation of Flavonoids -Aluminium chloride Colorimetric method

Quercetin served as a benchmark for measuring the number of flavonoids. Quercetin dilutions at 100, 200, 400, 500, 600, 800, and 1000 µg/mL were used to create calibration curves. Using the aluminium chloride complex-forming assay, 1 mL of the sample solution was combined with 4 mL of distilled water. Following a 5-minute incubation period, 300 µL of sodium nitrite and 300 µL of aluminium chloride were added, respectively. Finally, sodium hydroxide (two milliliters) was added.

Estimation of Alkaloid Content - Harborne's method

After combining the extract with 10% acetic acid in ethanol, it was left to stand for four hours. The extract was filtered and then concentrated to one-fourth of its original volume on a water bath. Ammonium hydroxide concentrate was added drop by drop till precipitation happened. After allowing the mixture to settle for three hours, the supernatant was disposed. Following a 0.1 M ammonium hydroxide rinse, the precipitates were dried, weighed, and cleaned(12). The following formula was used to determine the percentage of alkaloids:

$$Percentage\ of\ alkaloid = \frac{weight\ of\ sample}{Weight\ of\ alkaloids}\ X\ 100$$

Green Synthesis of *Phyla nodiflora* Silver Nanoparticles (Pn-Ag-Np)

Preparation of *Phyla nodiflora* Silver Nanoparticles

A 10 mL volume of the ethanolic extract (1 mg of extract dissolved in 10 mL of ethanol) was mixed with 90 mL of $AgNO_3$ solution (0.04 g dissolved in 100 mL of distilled water) and heated for 1 hour until a color change was observed. The solution was kept in the dark for 24 hours. The nanoparticles were purified by washing three times with sterile distilled water via centrifugation at 10,000 rpm for 10 minutes.

Characterization of *Phyla nodiflora* Silver Nanoparticles (Pn-Ag-Np)

The synthesised *Phyla nodiflora* Silver Nanoparticles (Pn-Ag-Np) were characterized for further analysis.

UVSpectroscopy

The reduction of Ag^+ to Ag^0 was monitored using a Perkin Elmer UV-Vis spectrophotometer (Lambda 365 model). Absorption scans were conducted over a wavelength range of 200 to 800 nm.

Particle Size Determination

The average mean diameter and size distribution of the nanoparticles were determined using Dynamic Light Scattering (Malvern Zetasizer). The dried nanoparticles

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were dispersed in water to obtain the necessary light scattering intensity.

Zeta Potential Measurement

The surface charge of Pn-Ag-Np was measured using a Malvern Zetasizer equipped with zeta cells and polycarbonate cells with gold-plated electrodes, using water as the medium for sample preparation. This measurement is crucial for assessing the stability of the nanoparticles.

Scanning Electron Microscopy (SEM) with EDAX

Scanning Electron Microscopy (SEM) was conducted to evaluate the surface morphology of *Phyla nodiflora* silver nanoparticles (Pn-Ag-Np). The nanoparticles were prepared by first drying the sample thoroughly to eliminate any moisture content. The SEM analysis provided high-resolution images of the surface features, morphology, and particle size distribution of the Pn-Ag-Np.

High-Resolution Transmission Electron Microscopy (HRTEM)

HRTEM analysis of Pn-Ag-Np was performed to evaluate the size, shape, and dispersion of the nanoparticles. The detailed structural properties and lattice fringes were observed, confirming the nanoscale dimensions and crystalline nature of the synthesized nanoparticles(13.)

Antioxidant Activity of Pn-Ag-Np

DPPH Assay for the synthesised Pn-Ag-Np

Various concentrations of standard ascorbic acid and samples (100, 200, 400, 800, and 1000 µg/mL) were prepared in distilled water. Equal volumes of different concentrations of ascorbic acid and DPPH were mixed and incubated at room temperature in the dark for 30 minutes. Absorbance was measured at 517 nm. The scavenging activity was calculated using the formula:

$$Scavenging\ activity\ (\%) = \frac{Ac - As}{AC}\ X\ 100$$

Nitric Oxide Scavenging Assay for the synthesised Pn-Ag-Np

After preparing a 3 mL reaction mixture in distilled water with different concentrations of standard ascorbic acid and samples (100, 200, 400, 800, and 1000 µg/mL) and sodium nitroprusside (10 mM in phosphate-buffered saline), it was incubated at 37°C for 4 hours. Griess reagent (0.5 mL) was added after incubation, and the absorbance was measured at 546 nm(14). The following formula was used to get the % inhibition:

$$Percentage\ inhibition = \frac{Abs\ control - Abs\ sample}{Abs\ control}\ X\ 100$$

Formulation of Pn-Ag-Np loaded Hydrogel

Hydrogels were formulated using carbopol-940, propylene glycol, methyl paraben, propyl paraben, and triethanolamine. Once the hydrogel base components were mixed, Pn-Ag-Np was added to the hydrogels, and the final volume was adjusted to 50 mL using distilled water. The formulations were stirred under magnetic stirring at 700 rpm for 1 hour at room temperature. Triethanolamine was then added dropwise with vigorous stirring until gel consistency and appropriate pH were achieved. Various formulations trials in Table 1 were prepared and evaluated by following parameters.

Evaluation Parameters for Pn-Ag-Np Loaded Hydrogel (Pn-Ag-Hg)

pH

A pH meter was used to measure each Pn-Ag-Hg formulation's pH. The standard deviation (SD) was determined after the experiments were carried out in triplicate.

Viscosity

The viscosity for each Pn-Ag-Hg formulation was determined by means of a Brookfield Viscometer. The trials were run three times, and the standard deviation was computed.

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Table 1: Formulation trials of Pn-Ag-Np hydrogel

Formulation Code	Pn-Ag-Np (g)	Carbopol (g)	Propylene Glycol (mL)	Methyl Paraben (g)	Propyl Paraben (g)	Triethanolamine (mL)	Distilled Water (mL)
F1	0.5	1	25	0.01	0.01	0.5	25
F2	1	1	25	0.01	0.01	0.5	25
F3	1.5	1	25	0.01	0.01	0.5	25
F4	2	1	25	0.01	0.01	0.5	25
F5	0.5	2	25	0.01	0.01	0.5	25
F6	1	2	25	0.01	0.01	0.5	25
F7	1.5	2	25	0.01	0.01	0.5	25
F8	2	2	25	0.01	0.01	0.5	25

Homogeneity

A visual inspection was used to assess the homogeneity of each Pn-Ag-Hg formulation. Aggregate content in the formulations was investigated. The trials were completed in triplicate, and the standard deviation was computed.

Spreadability

Every Pn-Ag-Hg formulation's spreadability was assessed. It was measured in terms of how long it took for two slides, subjected to a given weight, to slide off the gel that was placed between them. A spreadability (S) calculation was made.

$$S = \frac{M\ X\ L}{T}$$

Where, M = weight tied to upper slide, L = length of glass slide, T = time taken to separate the slides

Drug Content analysis by UV spectroscopy

Each Pn-Ag-Hg formulation was dissolved in a predetermined amount in 100 milliliters of pH 6.8 phosphate buffer. After filtering the mixture, phosphate buffer (pH 7.4) was used as the blank in a spectrophotometric analysis of the solution at 410 nm. The formulation trials were run through three times, and the standard deviation was calculated.

***In-vitro* drug permeation study**

The research adopted a diffusion cell apparatus that covered a receptor compartment with a membrane saturated in glycerol and used phosphate buffer as the dissolving media. To ascertain drug permeation over time, samples were examined while the medium was agitated at 50 rpm. On the basis of drug release profiles, the optimal formulation was chosen for additional assessment and research. The best formulation was chosen for more analysis and research.

Release Kinetics study for the for the optimized F4 formulation

The *In-vitro* release kinetics were evaluated for the optimized formulation to investigate the mechanism of drug release(15). The data were analyzed using the following mathematical models, such as zero order kinetics, first order kinetic, Higuchi kinetic, Kors Meyer - Peppa's Model and Hixson-Crowell cube root law

***In- vitro* cell line study using NHDF cell lines for the optimized F4 formulation**

MTT assay for the optimized F4 formulation

After being planted onto a 96-well plate at a density of 1 x 10^4 cells per well in 100 µL of DMEM media, Normal Human

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Dermal Fibroblast (NHDF) cells were allowed to develop for a full day. The medium was changed to several concentrations of the optimized F4 formulation (25, 50, 100, and 500 µg/mL) after the first incubation. After that, the plates were incubated for a full day more. After that, each well received 10 µL of the 5 mg/mL MTT reagent, and the plates were incubated for an additional 4 hours. The resultant purple formazan crystals were made soluble by filling every well—including the control wells—with 100 µL of dimethyl sulfoxide (DMSO) (without treatment). After giving the plates, a gentle shake in order to ensure complete mixing, they were left at room temperature in the dark for around half an hour. The absorbance was then measured with a microplate reader at 570 nm. Using the formula below, the percentage cell viability (CV) was determined:

$$CV\ \% = \frac{Absorbance\ of\ test\ sample}{Absorbance\ of\ control}\ X\ 100$$

In-vitro Wound Scratch Assay for the optimized F4 formulation

The migration rates of NHDF cells were assessed using the scratch assay method. Cells were seeded into each well of a 24-well plate at a density of 2 x 10^5 cells and incubated with complete medium at 37°C and 5% CO_2 until a confluent monolayer was formed. The monolayer cells were then scraped horizontally with a sterile P200 pipette tip to create a scratch. The debris was removed by washing the cells with PBS. Subsequently, the cells were treated with various concentrations of the optimized F4 formulation (10, 25, and 50 µg/mL) diluted in serum-free DMEM. Untreated cells served as the control, and cells treated with allantoin (50 µg/mL) served as the positive control.Images of the scratch, representing the wound, were captured at 0 hours using phase contrast microscopy at 50x magnification. After 24 hours of incubation with the F4 formulation, a second set of images was taken. The migration rate was determined by analyzing the images using "ImageJ" software to measure the percentage of the closed area compared to the initial wound area at 0 hours. An increase in the percentage of the closed area indicated cell migration(16). All experiments were performed in triplicate, and the data were recorded and analyzed statistically using GraphPad Prism version 10.

$$Wound\ Closure\ \% = \frac{Measurement\ at\ 0\ hour - Measure\ at\ 24\ hour}{Measurement\ at\ 0\ hour}\ X\ 100$$

Statical analysis

All experimental data were presented as mean ± standard deviation (SD). The MTT assay, *In-vitro* wound scratch assay, and hydrogel formulation parameters were each conducted in triplicate to ensure reliability and reproducibility. The statistical analysis was performed using GraphPad Prism version 10 software. One-way ANOVA followed by Tukey's post-hoc test was used to assess the statistical significance between different groups. The significance level was set at $p < 0.05$ for all analyses.

Results and discussion:

Extraction

The Percentage yield of the ethanolic extract was found to be 31.6% W/W.

Preliminary phytochemical screening

A thorough analysis of the phytochemical content of *Phyla nodiflora* 's ethanolic extract has revealed a wide range of bioactive chemicals. The rich chemical profile of this plant species is highlighted by the presence of alkaloids, proteins, saponins, starch, amino acids, steroids, terpenoids, tannins, flavonoids, anthraquinones, and glycosidesshown in Table 2. Because of these chemicals' noteworthy biological activity and possible pharmacological effects, *Phyla nodiflora* is an ideal choice for additional research in the fields of medicine and Pharmaceuticals.

Total Phenol content

The phenol content analysis of the ethanolic extract of *Phyla nodiflora* yielded

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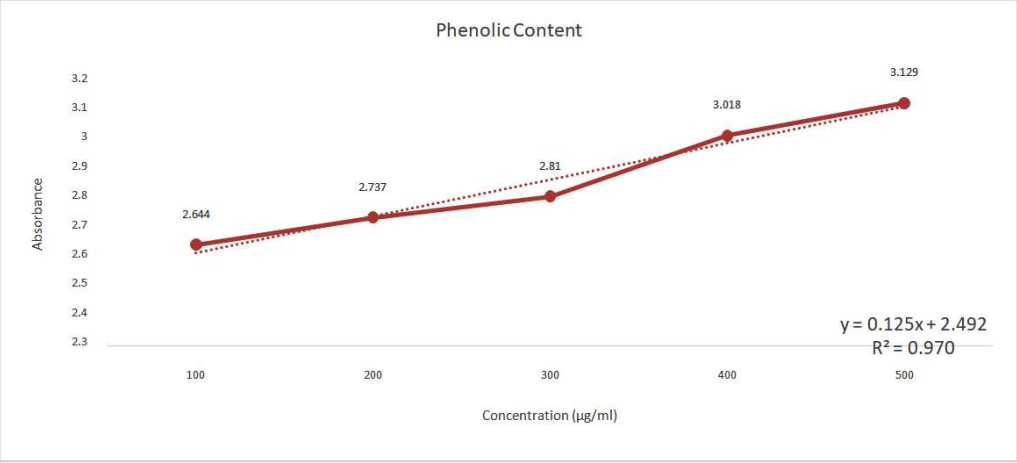
Table 2:Preliminary phytochemical screening of *Phyla nodiflora* ethanolic extract		
S. No	Phytochemical analysis	Report
1.	Alkaloids	+
2.	Proteins	+
3.	Saponins	+
4.	Carbohydrates	+
5.	Amino Acids	+
6.	Steroids	+
7.	Terpenoids	+
8.	Tannins	+
9.	Flavonoids	+
10.	Anthraquinones	+
11.	Glycosides	+



Graph 1: The calibration curve of Gallic acid for determination of Phenol content in the *Phyla nodiflora* extract

value of 0.16 mg GAE/g, calculated using the regression equation y = 0.1251x + 2.49. The graph was illustrated in Graph 1. This finding underscores the significant presence of phenolic compounds in the sample, crucial for their antioxidant properties and potential health benefits

Total Tannin content

The tannin content estimation of the ethanolic extract of *Phyla nodiflora* revealed a value of 4.00 mg TE/g, derived from the regression equation y = 0.6495x + 0.7357. The graph was shown in Graph 2. This indicates a substantial presence of tannins in the sample, essential for various biological activities and applications.

Total Flavonoid content

The analysis of flavonoid content of the ethanolic extract of *Phyla nodiflora* resulted in a value of 0.749 mg QE/g, obtained from the regression equation y = 0.0997x + 0.0693. The graph was illustrated in Graph 3. This highlights the significant

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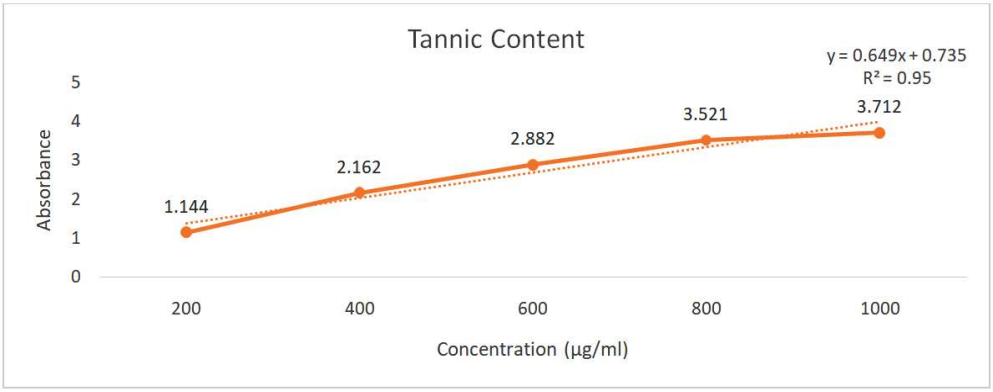
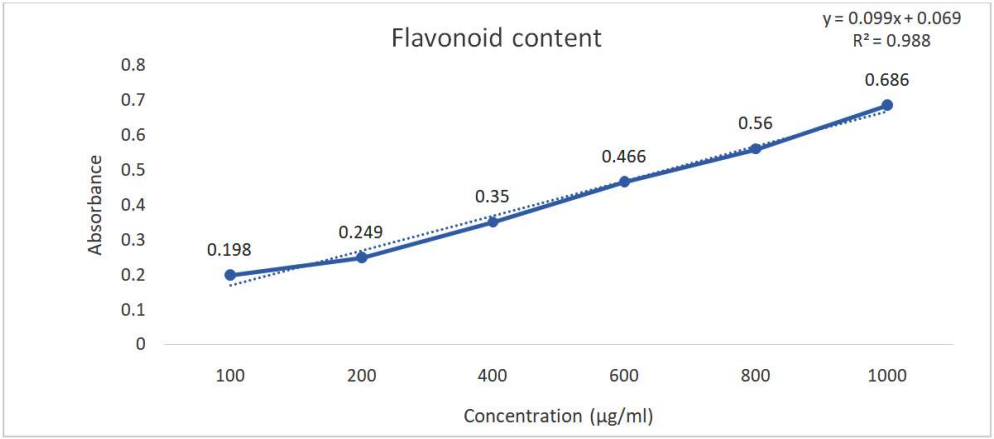


Graph 2: The calibration curve of Tannic acid for determination of Tannin content in the *Phyla nodiflora* extract



Graph 3: The calibration curve of Quercetin for determination of flavonoid content in the *Phyla nodiflora* extract

concentration of flavonoids in the sample, known for their diverse biological activities and health-promoting effects

Total Alkaloid Content

The significant alkaloid content was found to be 23.5% w/w in the ethanolic extract of *Phyla nodiflora* highlights its potential pharmacological importance.

Characterization of *Phyla nodiflora* Silver Nanoparticles (Pn-Ag-Np)

UV Spectroscopy

The spectrum's detected UV peak at 410 nm suggests the existence of silver nanoparticles Figure 1. This peak relates to silver nanoparticles' surface plasmon resonance (SPR) absorption feature, in which incoming light causes conduction electrons on the nanoparticle surface to oscillate collectively. Because of their size and shape, silver nanoparticles usually exhibit an SPR peak in the 400–450 nm range, which verifies the presence of silver nanoparticles in the solution.

Zeta size and zeta potential

The zeta size measurement of 76.53 nm suggests the average size of the silver nanoparticles in the solution. This parameter

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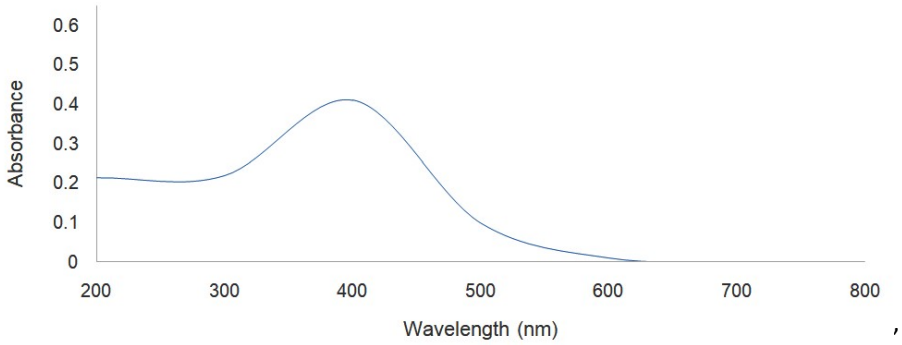

Figure 1: UV Spectroscopy of synthesis silver nanoparticle

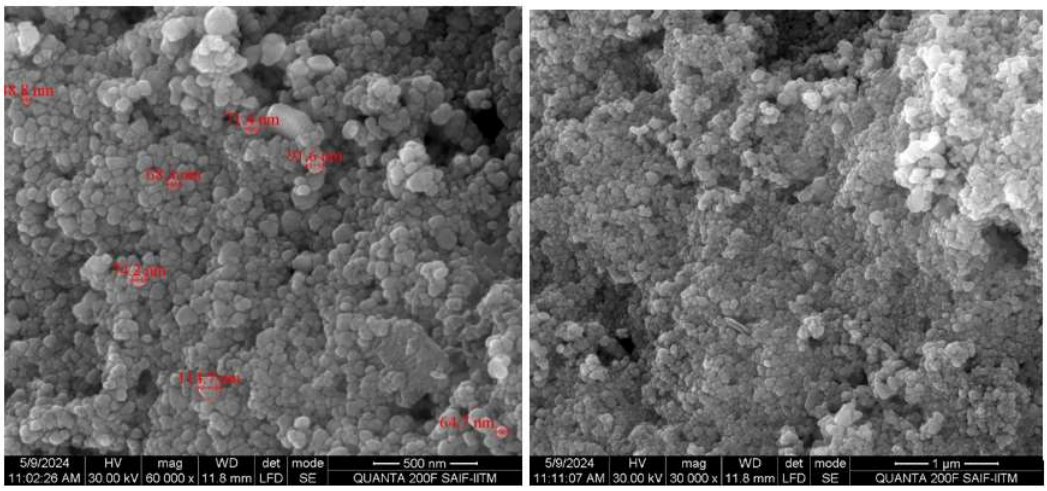

Figure 2: SEM analysis of synthesised Pn-Ag-Np

is crucial for understanding the physical dimensions of the nanoparticles. The negative zeta potential value of -30.72 mV indicates the surface charge of the nanoparticles. The high negative potential suggests good stability, as the nanoparticles repel each other, inhibiting aggregation.

SEM analysis

The SEM image, captured at 60,000x magnification, reveals the nanostructure of the silver nanoparticles synthesized from the ethanolic extract of Phyla nodiflora. The nanoparticles, with diameters ranging from 64.7 nm to 114.7 nm, are uniformly distributed within the matrix shown in figure 2. Analysis conducted using a QUANTA 200F at SAIF-IITM demonstrates the potential of this silver nanoparticles for wound healing applications due to its enhanced antimicrobial properties.

EDAX

The EDAX analysis of the synthesized nanoparticle indicates that the primary element present is silver (Ag), with a weight percentage (wt%) and atomic percentage (at%) both being 100% shown in Figure 3A. This suggests the nanoparticles are predominantly composed of silver, with

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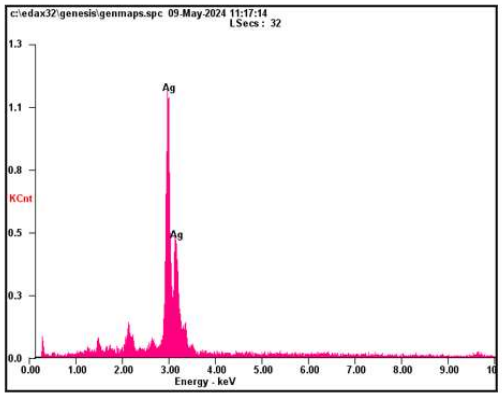

Element	Wt%	At%
AgL	100.00	100.00
Matrix	Correction	ZAF

Figure 3A: EDAX analysis of synthesised Pn-Ag-Np

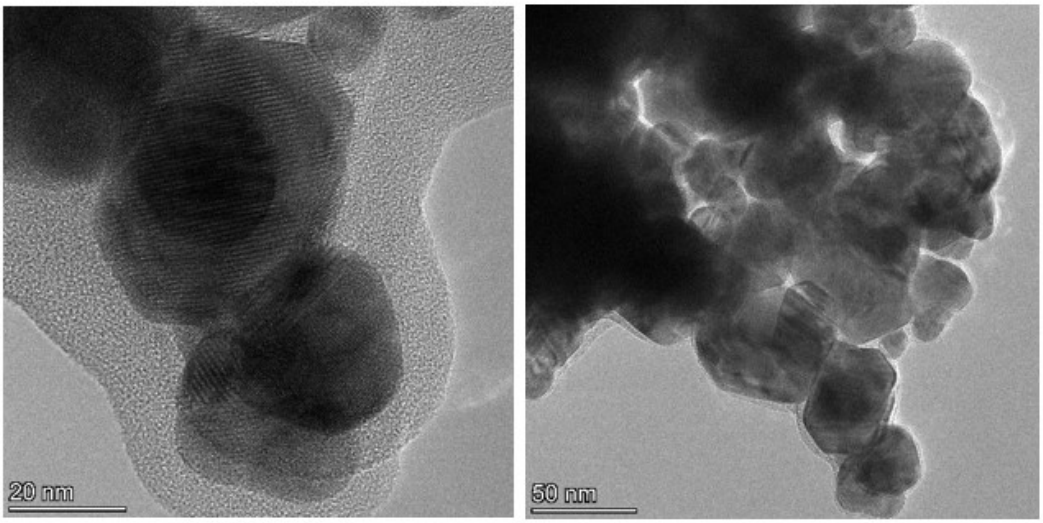

Figure 3B: HRTEM analysis of synthesised Pn-Ag-Np

no significant impurities detected. The matrix weight percentage correction and At% ZAF values further confirm the purity and elemental composition, ensuring the accuracy of the elemental analysis

HRTEM

TEM analysis of the silver nanoparticles synthesized from the ethanolic extract of Phyla nodiflora shows well nanostructures within the matrix. The high-resolution images, taken at scales of 50 nm and 20 nm, confirm the successful incorporation and uniform dispersion of the nanoparticles shown in Figure 3B nanostructures are critical for the wound healing offering improved antimicrobial activity and biocompatibility.

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Antioxidant Activity of Pn-Ag-Np

DPPH Assay for synthesisedPn-Ag-Np

The DPPH assay results indicate that the synthesized nanoparticles exhibit strong antioxidant activity, which increases with concentration. The test sample's scavenging activity, reaching 95.5% at 1000 µg/mL, reflects significant free radical neutralization capability shown in Graph 4. This concentration-dependent antioxidant activity makes these nanoparticles suitable for applications in pharmaceuticals and cosmetics where oxidative damage prevention is essential. The slight variations between the test and standard values are within acceptable ranges, confirming the nanoparticles' effectiveness.

Nitric Oxide scavenging of synthesisedPn-Ag-Np

The synthesized nanoparticles demonstrated notable NO scavenging activity, with 77.89% inhibition at 100 µg/mL and 98.59% at 1000 µg/mL shown in Graph 5. These high levels of activity are beneficial for medical applications aimed at controlling inflammation and oxidative stress. The potent NO scavenging properties suggest potential use in developing anti-inflammatory drugs and treatments for conditions associated with excessive NO production.

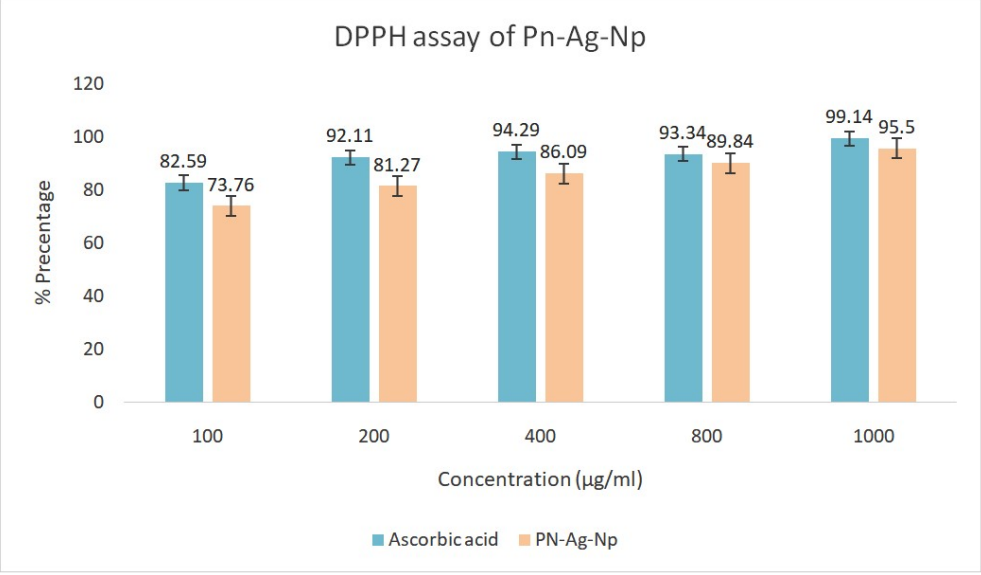
Evaluation parameter of formulated*Phyla nodiflora* silver nano loaded Hydrogel (Pn-Ag-Hg)

pH

The pH of hydrogel formulations is crucial as it affects the stability and compatibility with the skin. Formulation F4 exhibited a pH of 7.0 ± 0.1, which falls within the acceptable range for topical applications. The slightly higher pH can enhance the solubility of the active ingredients and promote better absorption into the skin. The results in Table 3 demonstrate that F4 maintained a consistent pH level, indicating stability and suitability for dermatological use.

Viscosity

Viscosity plays a critical role in the spreadability and application characteristics of hydrogels. Formulation F4 demonstrated a



Graph 4: DPPH assay of Pn-Ag-Np

Phyla Nodiflora Derived Silver Nanoparticles

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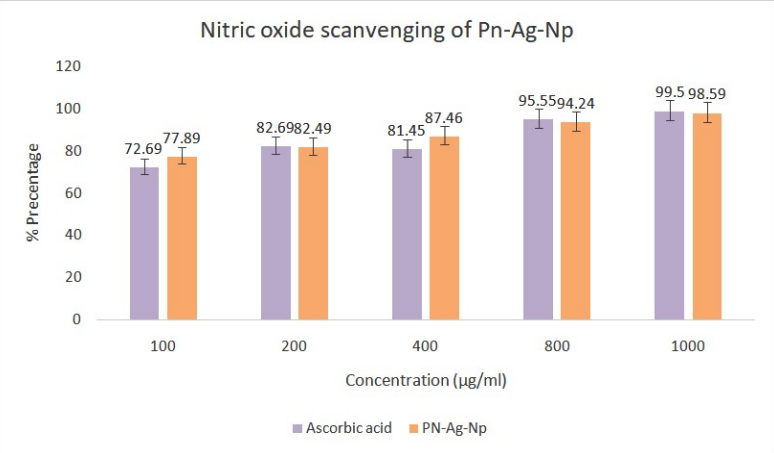

Graph 5: Nitric oxide scavenging of Pn-Ag-Np

Table 3: Physicochemical evaluation of the Pn-Ag-Hg for various trials

Parameter	F1	F2	F3	F4	F5	F6	F7	F8
pH	6.8 ± 0.1	6.9 ± 0.1	6.9 ± 0.1	7.0 ± 0.1	6.7 ± 0.1	6.8 ± 0.1	6.8 ± 0.1	6.9 ± 0.1
Viscosity (cP)	1500 ±50	1520 ±55	1530 ± 60	1550 ±65	1600± 70	1620 ± 75	1640 ± 80	1660 ± 85
Homogeneity	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Spreadability (g cm/s)	20 ± 1	21 ± 1	22 ± 1	23 ± 1	18 ± 1	19 ± 1	20 ± 1	21 ± 1
Drug Content (%)	95 ± 2	96 ± 2	97 ± 2	98 ± 2	94 ± 2	95 ± 2	96 ± 2	97 ± 2

viscosity of 1550 ± 65 cP, which is suitable for easy application and adherence to the skin surface. The viscosity values across formulations F1 to F8 Table 3 show that F4 falls within the desired range, ensuring optimal handling and effectiveness during application.

Homogeneity

Homogeneity is essential to ensure uniform distribution of active ingredients within the hydrogel matrix. All formulations, including F4, exhibited uniform consistency without visible aggregates or phase separation, the results are shown in Table 3. This uniformity indicates that F4 can deliver consistent dosing and efficacy, crucial for therapeutic applications.

Spreadability

Spreadability influences patient compliance and ease of application. Formulation F4 demonstrated a spreadability of 23 ± 1 g cm/s, indicating good glide over the skin surface. The results in Table 3 show that F4 achieved superior spreadability compared to other formulations, suggesting enhanced user experience and better coverage of the affected area.

Drug Content

The drug content in hydrogels determines the amount of active ingredient available for therapeutic action. Formulation F4 exhibited a high drug content of 98 ± 2%, indicating efficient encapsulation and stability

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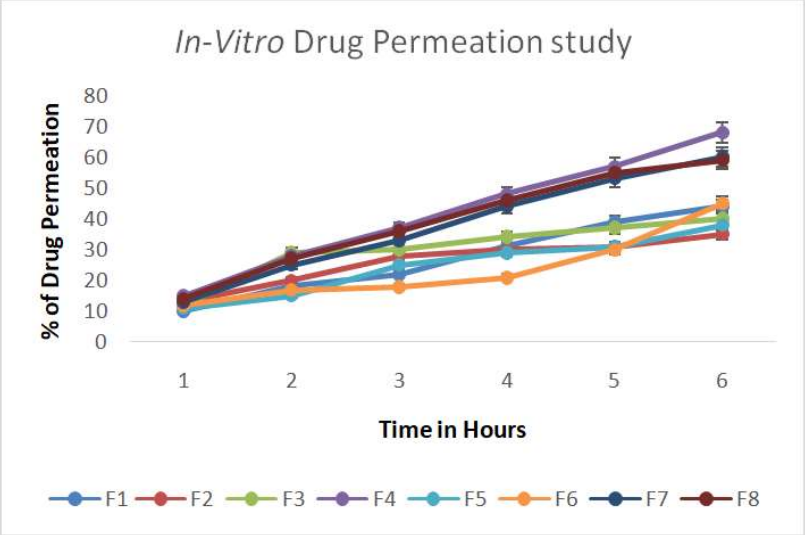

Graph 6: *In-vitro* drug permeation study for various formulation trials in 6 hours

of Pn-Ag-Np within the hydrogel matrix. The data presented in Table 3 highlight that F4 maintained consistent drug content, ensuring reliable and effective delivery of therapeutic agents.

Drug Release

The drug release profile is crucial for achieving therapeutic efficacy over time. Formulation F4 demonstrated a cumulative drug release of 78 ± 5% over the study period, indicating sustained release characteristicsshown in Table 3. The higher drug release observed in F4 suggests that it can provide prolonged therapeutic effects, which is beneficial for chronic wound management and healing acceleration.

***In-vitro* drug permeation study**

The drug release profile is crucial for achieving therapeutic efficacy over 6 hours. Formulation F4 demonstrated a cumulative drug permeation release of 68 ± 5% over the study period, indicating sustained release characteristics in Graph 6. The higher drug release observed in F4 hence it is choosed as the optimized formulation and also suggests that it can provide prolonged therapeutic effects, which is beneficial for chronic wound management and healing acceleration.

Table 4: Release kinetics of Optimized F4 formulation

Release Kinetic Model	r^2
Zero-order Kinetics	0.943
First-order Kinetics	0.975
Higuchi Model	0.986
Korsmeyer-Peppas Model	0.988
Hixson-Crowell Model	0.964

Release Kinetics for Optimized Formulation (F4)

The release kinetics of formulation F4 were analyzed using various mathematical models to understand the mechanism of drug release. The r^2 values for each model are summarized below Table 4:

Based on the r^2 values, the drug release from the optimized formulation F4 follows the Korsmeyer-Peppas model (0.988) and Higuchi model (0.986), indicating that the release mechanism is predominantly diffusion-controlled.

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In- vitro cell line study using NHDF cell lines for the optimized F4 formulation

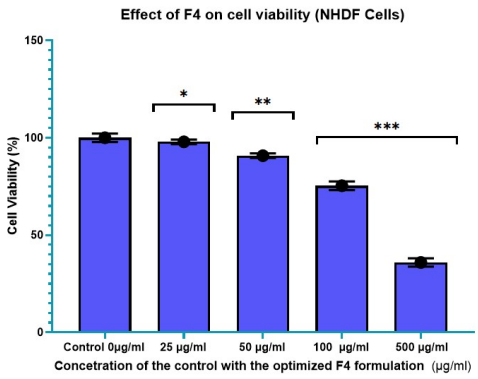
MTT assay for the optimized F4 formulation

The cytotoxicity of the optimized Pn Ag-Hg hydrogel formulation (F4) was assessed using the MTT assay on NHDF cells, as depicted in Graph 7. The control group (0 µg/mL) exhibited a cell viability of 100% ± 5%. At a concentration of 25 µg/mL, cell viability slightly decreased to 95% ± 4%, with a statistically significant reduction compared to the control ($p = 0.02$). Increasing the concentration to 50 µg/mL further reduced cell viability to 90% ± 6%, with a higher significance level ($p = 0.01$). A more pronounced cytotoxic effect was observed at 100 µg/mL, where cell viability decreased to 80% ± 7% ($p = 0.001$). The highest concentration of 500 µg/mL resulted in a substantial drop in cell viability to 40% ± 10%, with a highly significant difference compared to the control ($p < 0.001$). These findings indicate a clear dose-dependent cytotoxic effect of the F4 formulation, which is critical for determining its safe therapeutic range.

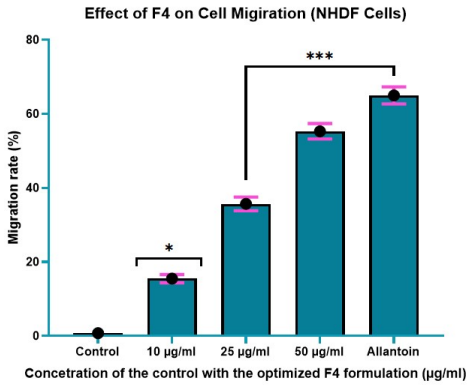
In-vitro Wound Scratch Assay for the optimized F4 formulation

The scratch wound healing assay assessed the wound closure capability of NHDF cells treated with various concentrations of the F4 formulation are shown in Figure 4 and Graph 8. Figure 4 illustrates the wound closure at o hours and 24 hours post-treatment. Control cells showed minimal wound closure after 24 hours. In contrast, treatment with F4 at 10 µg/mL, 25 µg/mL, and 50 µg/mL resulted in dose-dependent increases in wound closure. In graph 8 the control group (0 µg/mL) had a baseline migration rate of 0% ± 1%. Treatment with 10 µg/mL of F4 increased the migration rate to 15% ± 3%, with a statistically significant difference compared to the control ($p = 0.05$). A further increase in concentration to 25 µg/mL resulted in a migration rate of 30% ± 4%, showing a highly significant difference from the control ($p = 0.001$). At 50 µg/mL, the migration rate rose significantly to 50% ± 5% ($p < 0.001$). The positive control, Allantoin, produced a migration rate of 60% ± 3%, which was also highly significant compared to the control ($p < 0.001$). These results demonstrate that F4 enhances cell migration in a dose-dependent manner, suggesting its potential utility in wound healing and tissue regeneration applications.

The research effectively showcased the formulation of a phytopharmaceutical hydrogel that integrated silver nanoparticles from *Phyla nodiflora* that were green synthesized. The thorough phytochemical



Graph 7: Effect of F4 on cell viability



Graph 8: Effect of F4 on Cell Migration (NHDF Cells)

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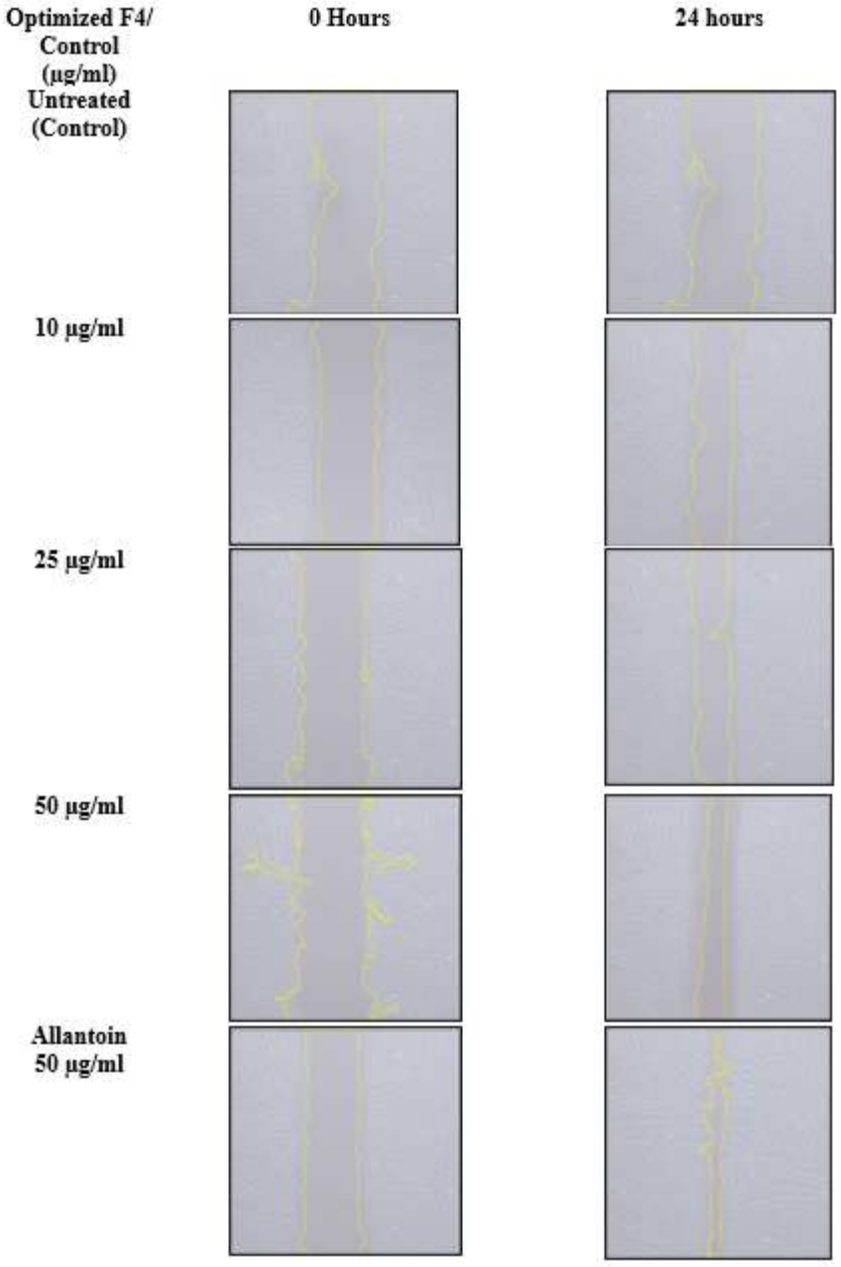

Figure 4: The *In-vitro* wound scratch assay using NHDF Cells

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investigation verified the existence of bioactive substances, which are essential for the hydrogel's medicinal potential. The nanoparticles' proper size, stability, and shape were revealed by characterization, which guaranteed their effectiveness in the hydrogel matrix. Assessing the hydrogel's physicochemical characteristics and conducting *In-vitro* studies validated its appropriateness for wound healing purposes, especially in the treatment of persistent wounds.

Conclusion

Promising outcomes in wound healing have been observed with the hydrogel matrix incorporating *Phyla nodiflora* silver nanoparticles. The anti-inflammatory and antioxidant characteristics of the ethanolic extract promote wound healing. The hydrogel shows promise as a viable alternative for treating chronic wounds, and its environmentally friendly manufacturing further augments its therapeutic potential.

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Conflict of Interest

The corresponding author states that there is no conflict of interest

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Enhancing Breast Cancer Treatment: Investigating the Influence of Polymer Ratios on Luteolin-Loaded TPGS/Poloxamer Micelles

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Abstract

Luteolin is a widely studied flavonoid recognized for its ability to sensitize multidrug-resistant cells and anti-cancer properties. However, its clinical applications is hindered by challenges arising from its limited solubility and bioavailability. To address this issue, a synergistic approach combiningluteolin with vitamin E TPGS (TPGS) and poloxamer (Pol) was explored to boost tumour apoptosis and suppress P-glycoprotein. This study aimed to optimize luteolin-loaded TPGS/Pol micelles developed using the film hydration method, followed by lyophilization. Various key factors including the drug: polymer ratio and the TPGS: Pol ratio were examined, with encapsulation efficiency (EE) assessed using a UV-Vis spectrophotometer and particle size measured via dynamic light scattering (DLS). The results revealed that the optimized micelle composed of 1:5 drug: polymer ratio and a TPGS: Pol ratio of 3:1, exhibited a particle size below 40 nm, along with EE of 90%. Additionally, there was a notable increase of 459-fold in the solubility of luteolin-loaded micelle in comparison to pure luteolin in water. The TPGS/Pol micelles demonstrated a critical micelle concentration (CMC) of 0.0008 mg/ml, andrelease studies indicated sustained release behaviour for luteolin-loaded micelles. In conclusion, this study has presented the feasibility of TPGS/Pol micelles in improving the therapeutic potential of luteolin, showcasing improvements in EE, particle size, solubility and sustained release behaviour.

Keywords: Luteolin; TPGS; Poloxamer; Optimization; Micelle; Breast cancer.

Introduction

The micellar drug delivery system stands out as a versatile and innovative technology designed to transport poorly soluble drugs to specific target sites. In addition to enhancing the solubility of lipophilic drugs, this system also improves drug bioavailability, targeting precision and release profiles(1,2). Many studies have explored the efficiency of micellar drug delivery systems, particularly in the context of cancer treatment. Conventional chemotherapeutic drugs currently available in the market compromise the quality of life for patients undergoing chemotherapy, owing to their mechanism targeting actively growing cells. However, these drugs inadvertently affect not only tumor cells but also actively growing normal cells, resulting in undesired effects such as alopecia, anemia, and neutropenia(3).

The applicability of micellar drug delivery systems in cancer studies is underscored by their intrinsic targeting

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properties, whether actively or passively achieved, contributing to enhanced drug efficacy and reduced side effects(4,5). Luteolin, a natural bio-compound belonging to the flavone class found in fruits and vegetables like parsley and artichokes, has demonstrated antioxidant, anti-inflammatory, and anti-cancer properties(6,7). Moreover, luteolin shows cytotoxic effects on various cancer cell lines, including lung, colorectal, breast and liver cancer. It demonstrates the ability totrigger cell death, arrest cell cycle progression and inhibit metastasis(8).

Despite its advantageous properties, luteolin's poor solubility in water limits its bioavailability and efficacy, necessitating innovative approaches to fully harness its potential. For instance, luteolin encapsulated in MPEG-PCL micelles has shown a lower inhibition concentration (IC_{50}) and improved pharmacokinetic parameters in contrast to free luteolin(9). This highlights the significance of encapsulating luteolin into micelle: to improve solubility, bioavailability and efficacy.

Vitamin E TPGS, also refer toD-α-tocopheryl polyethylene glycol succinate (TPGS), represents an amphiphilic triblock co-polymer recognized for its multifunctional properties including serving as a solubilizer, surfactant, and additional attribute(10). Extensive research has focused on its application in facilitating drug micellization for cancer therapeutics, leveraging its distinct features such as acting as an inhibitor of P-glycoprotein and inducing apoptosis (Luiz et al., 2021). Similarly, Poloxamer 407, or Pluronics F127, has been explored as a constituent in the construction of encapsulation complexes within micellar nanocarriers for cancer treatment, owing to its amphiphilic nature. Reports indicate that Pluronics exhibit P-gp inhibition in drug-resistant cancer cells(12). Nonetheless, literature suggests that Poloxamer 407's impact on chemo-resistant tumor cells is limited due to its hydrophilicity (HLB:22)(13). To address this limitation, hydrophilic Pluronics have been utilized to enhance circulation time and evade the reticulo-endoplasmic system (RES) within the bloodstream. Therefore, the theoretical synergistic potential of combining these two copolymers with luteolin holds promise in combating breast cancer cells.

In this study, we aim to develop a micellar system comprised of TPGS and Poloxamer 407 (Pol) to encapsulate luteolin and enhance its solubility in water. Our hypothesis proposes that the polymer/drug and the polymer/polymer ratio will significantly influence the physicochemical properties of the micelle, as suggested by prior research. The objectives of this study include investigating the impact of polymer/polymer and polymer/drug ratios on micelle properties, developing micelle with smaller particle size and increased encapsulation efficiency, and characterizing the solubility and release profile of the optimized micelle.

Materials and Methods

Materials

Luteolin and vitamin E TPGS (tocofersolan) were supplied by MedChem Express (Monmouth Junction, NJ, USA). Poloxamer 407 (Pol) was obtained from Sigma-Aldrich (St. Louis, MO, USA), while ethanol was acquired from R&M Chemicals SdnBhd (Subang, Malaysia), while

Development of luteolin-loaded micelle

The development of luteolin-loaded micelles was conductedthrough thin film hydration method, following the procedure outlined in a previous study(14). Luteolin, TPGS and Polwere mixed in ethanol until achieving homogeneity. After removing the solvent utilizinga rotary evaporator, the resulting thin film underwent overnight vacuum-drying. Subsequently, the thin film was immersed in 10 mL of water with continuous stirring and heating until a micellar solution formed. The solution was then subjected to centrifugation and filtration, followed by freeze-drying to produce a solid micellar cake powder, which was kept at 4 °C until further analysis.

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Optimization of drug: Polymer ratio and TPGS: Pol ratio

The impact of drug: polymer ratio and TPGS: Pol ratio of luteolin-loaded micelleson encapsulation efficiency (EE) was explored. The drug: polymer ratio was predefined at 1:2.5, 1:5 and 1:7.5, while the TPGS: Pol ratio was adjusted to 4:0, 3:1, 1:3 and 0:4. The resulting micellar solutions underwent freeze-drying and kept at -4 °C for EE assessment. The micellar cake powder demonstrating optimized EE was then selected for particle size analysis.

Encapsulation efficiency assessment

The method for assessing EE was adapted from Patra et al.(14).A total of 1 mg of micellar cake powder was mixed with 5 mL of ethanol for micelle disruptionto facilitateluteolin release. The absorbance was assessed at a wavelength of 350 nm after dilution with ethanol. Triplicate measurements were performed, and the data are presented as mean ± standard deviation (SD). The percentage of EE was calculated using the following equation:

$$\%\mathrm{EE} = \frac{\text{Luteolin weight in micelle}}{\text{Initial weigth of luteolin}} \times 100$$

Particle size determination

The size of the micelles was measured using dynamic light scattering (DLS) with a Litesizer 100 (Anton Paar, Graz, Austria). The micellar cake powder was dissolved at a concentration of 1 mg/mL and diluted 10 times, followed byfiltrationutilizing a 0.22 µL syringe filter before being transferredinto the cuvette. The measurements were taken at a detection angle of 175°, with the temperature maintained at 25 °C.

Transmission electron microscopy

The Libra 120 Transmission electron microscopy (TEM, Carl Zeiss, Jena, Germany) was employed to reveal the surface morphology of the micelles and to confirm theirparticle size. A copper grid stained with a 2% phosphotungstic acid solution was deposited with a drop of micellar solution and observed under TEM after drying. This step provided additional insights into the micelle's structural characteristics.

Solubility Study

The solubility study was conducted following the method outlined in previous published literature(14), with slight modifications.Distilled water at 25 °C was added with an excess amount of luteolin powder and stirred at 120 rpm in a shaker incubatorfor 72 hours. After centrifugation and filtration through a 0.22 µm syringe filter, 5 mL of ethanol solution was added to 1 mL of the supernatantfor micelle disruption. The absorbance was measuredusing a UV spectrophotometer (Uviline 9400, Secomam, Mainz, Germany)at a wavelength of 350 nm.

Critical micelle concentration determination

The CMC of micelle was assessed using iodine as a hydrophobic probe(Patra et al., 2018; Saxena & Hussain, 2012).A potassium iodide/iodinestandard solution was mixed the serially diluted blank optimized micelle solution with concentrations ranging from 0.1% to 0.000001%. The mixtures were then placed in the dark at room temperature for 12 hours. Absorbance was measured using a UV-Vis spectrophotometer (Uviline 9400, Secomam, Mainz, Germany)at a wavelength of 366 nm, and the CMC was determined as the polymer concentration corresponding to an increase in absorbance, plotted against log polymer concentration.

***In-vitro* drug release study**

Dialysis method was performed to determine and compare the release kinetics of bothluteolin-loaded micelles and free luteolin (14,16).Dialysis membrane bags with sealed ends were separately filled with 1 mg of lut-loaded micelle and free luteolin in ethanol. These bags were then placed into 100 mL release media consisting of 0.5% Tween 80 in phosphate-buffered saline solution of pH 7.4 at 37 °C under 100 rpm of horizontal shaking.

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A total of 1 mL of aliquot of therelease media were withdrawn at predefined intervals, and the absorbance was assessed using a UV-Vis spectrophotometer at a wavelength of 350 nm. Concurrently, 1 mL of fresh release media was replenished each time an aliquot was withdrawn. To simulate the microenvironment of tumour cells, the release kinetic of luteolin-loaded micelles was also assessed in release media of pH6.8, as intracellular tumour cell pH typically ranges from 6.7 to 7.1 (17,18). This comprehensive investigation enables a thorough understanding of the drug release dynamics under conditions that mimic both physiological and tumour microenvironments.

Statistical analysis

The data are expressed as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) and Tukey's post hoc multiple comparisons for significance were performedusing JASP SOFTWARE (version 0.16.1). A significance level of $p < 0.05$ was considered statistically significant.

Results and Discussion

The impact of drug: Polymer ratio and polymer/polymer ratio on encapsulation efficiency

EE in nanoparticles is regarded as their capability to efficiently encapsulate and preserve bioactive compounds, proteins or drugs within their structure. This parameter holds significance in determining the efficacy and stability of delivery systems based on nanoparticles. The selection of materials in nanoparticle formulation stands out as a crucial factor influencing encapsulation efficiency. In this context, Table 1 presents that the mixed polymeric micelle outperformed its individual polymeric micelle counterparts with notably higher encapsulation efficiency recorded. This observation aligns with the findings of Chang et al. (19), wherebythe mixed micelles of PEGMEMA 12/PS 595 loaded with curcumin exhibited greater EE than single micelles comprising of PEO–PCL.

Table 1 demonstrates the EE of TPGS/Pol micelles, ranging from 74.3 nm to 92.3 nm. Among the various drug: polymer ratios (1:2.5, 1:5, and 1:7.5), ratios 1:5 (b) and 1:7.5 (c) exhibited higher EE compared to 1:2.5 (a). Particularly noteworthy is the substantial increase in EE at TPGS: Pol ratio 3:1 (B), surpassing other ratios and individual micelles significantly. This trendsuggests that a higher polymer-to-drug ratio correlates with increased EE. Consequently, TPGS: Pol ratio 3:1 emerged as the optimized ratio due to its superior EE compared to other ratios.

The formulations (Bb) and (Cb) were identified as optimized micelles owing to the greater EE obtained. Despite the negligible difference in EE between these two formulations, formulation Bb was selected for subsequent characterization studies. While a previous study proposed that higher EE holds promise in delivering active compounds by enhancing their bioavailability(20), other factors like cost and reproducibility werealso

Table 1: The encapsulation efficiency of luteolin-loaded tpgs/poloxamer micelle with different drug: polymer ratio and polymer: polymer ratio

	Encapsulation Efficiency (%)		
Drug: polymer ratio	1: 2.5 (a)	1: 5 (b)	1: 7.5 (c)
TPGS: Pol ratio			
4:0 (A)	78.6 ± 0.6	80.3 ±0.5	86.2 ± 0.5
3:1 (B)	85.5 ± 1.0	90.7 ± 0.9	92.3 ± 0.6
1:3 (C)	77.7 ± 0.2	79.9 ± 0.3	77.4 ± 0.5
0:4 (D)	77.0 ± 0.7	78.5 ± 0.4	74.3 ± 0.4

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taken into consideration during the selection of an optimized micelle. Micelle(Bb), utilizing a lesser amount of polymers, allows for the production of a larger quantity of micelles for subsequent tests. This strategic selection aligns with the goal of optimizing both performance and resource utilization in nanoparticle-based delivery systems.

The impact of drug: Polymer ratio and polymer: polymer ratio on particle size

In Table 2, the particle sizes of the optimized TPGS: Pol ratio was examined across various drug: polymer ratios. The recorded particle sizes range from 26.72 to 28.65 nm. Notably, the micelle with a drug: polymer ratio of 1:5 (b) exhibits the smallest particle size in comparison to ratios 1:2.5 (a) and 1:7.5 (c). The particle size analysis revealed significant variations among the micelles with different TPGS: Pol ratios, particularly in the 4:0 and 0:4 configurations, as shown in (Table 3). Notably, the micelle with TPGS: Pol ratio of 4:0 exhibited a considerably larger size than the 0:4 counterpart, indicating that TPGS contributes to the formation of smaller micelles compared to Pol. Intriguingly, the combination of both polymers resulted in a larger-sized micelle compared to each polymer individually, suggesting a synergistic effect in micelle size modulation.Significant differences in particle size were also noted between micelles with TPGS: Pol ratios of(Bb) and (Cb), further emphasizing that (Bb) represents the optimized micelle configuration. Consequently, this optimized micelle (Bb) was selected for subsequent tests and characterizations.

Table 2: The particle size of optimized micelle in comparison with other micelles at the same TPGS:Pol ratio (3:1), but different drug: polymer ratio

Drug: Polymer Ratio	Particle Size (nm)
1: 2.5 (a)	26.72 ± 1.55
1: 5 (b)	24.57 ± 0.61
1: 7.5 (c)	28.65 ± 1.32

This observed increase in size, as shown in Table 4, could be attributed to the hydrophilicity of poloxamer 407. In agreement with findings by Wei et al.(21) and Fares et al.(22), the presence of hydrophilic head from poloxamer 407 might contribute to the enlargement of micellar size. Moreover, the proportion of the hydrophilic polymer may influence micelle size, as supported by literature indicating that a lower concentration of hydrophilic polymer tends to reduce micelle size.

This finding establishes the drug: polymer ratio of 1:5 as the optimized configuration, and the formulation (Bb) will be utilized in the subsequent section to refer to the optimized micelle (TPGS: Pol ratio 3:1; drug: polymer ratio 1:5).

The typical size range of nanoparticle is between 1 to 1000 nm, a crucial characteristic with significant implications for theirproperties and behaviour(23), specifically regarding their ability to enhance permeability and retention to improve the penetration of tumour cell. Generally, the particle size of

Table 3: The particle size of optimized micelle in comparison with other micelles at the same drug: polymer ratio (1: 5), but different polymer: polymer ratio

Polymer: Polymer Ratio	Particle Size (nm)
4: 0	18.18 ± 1.01
3: 1	24.57 ± 0.61
1: 3	27.65 ± 1.11
0: 4	22.56 ± 0.66

Table 4: The solubility of luteolin when loaded into micelle in water in comparison with free luteolin in water

	Solubility in water (ug/ml)
Luteolin in water	30.67
Luteolin-loaded micelle in water	2594.02

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micelle must be below 200 nm in order for it to effectively reside within a tumour's blood vessels. The range of nanoparticle sizes conducive to the EPR effect generally falls between 1 to 400 nm (Chentamara et al., 2019).However, if the micelle's size exceeds 200 nm, there is a risk of elimination from the body through the reticuloendothelial system (RES)(24). Thus, thorough monitoring of nanoparticle size is vital to ensure the optimal delivery and efficiency of the drug within the tumour site.

The TEM image shown in Figure 1 illustrates the spherical shape of the micelle, characterized by distinct light grey and black layers, which corresponding to the hydrophilic and hydrophobic regions, respectively. This visualization supports our hypothesis regarding the dissolution and encapsulation of luteolin within the hydrophobic region of the micelle.

Solubility study

The water solubility of luteolin was 30.67 µg/mL, but significantly increased to 2594.02 µg/mL when encapsulated within a TPGS/Pol micelle. This represents a 459 times increase in solubility compared to its solubility in pure water. This phenomenon is further evident by visually examining the solution's physical appearance. In the absence of micelle encapsulation, the presence of luteolin leads to sedimentation, emphasizing the effectiveness of the micelle in preventing such precipitation.This observation highlights the efficacy of the TPGS/Pol micelle in enhancing the solubility of hydrophobic drugs likeluteolin in aqueous environments.

CMC determination

TheCMC was established by monitoring the abrupt rise in absorbance of the KI/I2 solution across diverse dilutions of the optimized blank micelle. The point of CMC determination was identified as the intersection between the steady absorbance reading and the sudden increase in absorbance. As illustrated in Figure 2, the CMC was precisely determined at a log

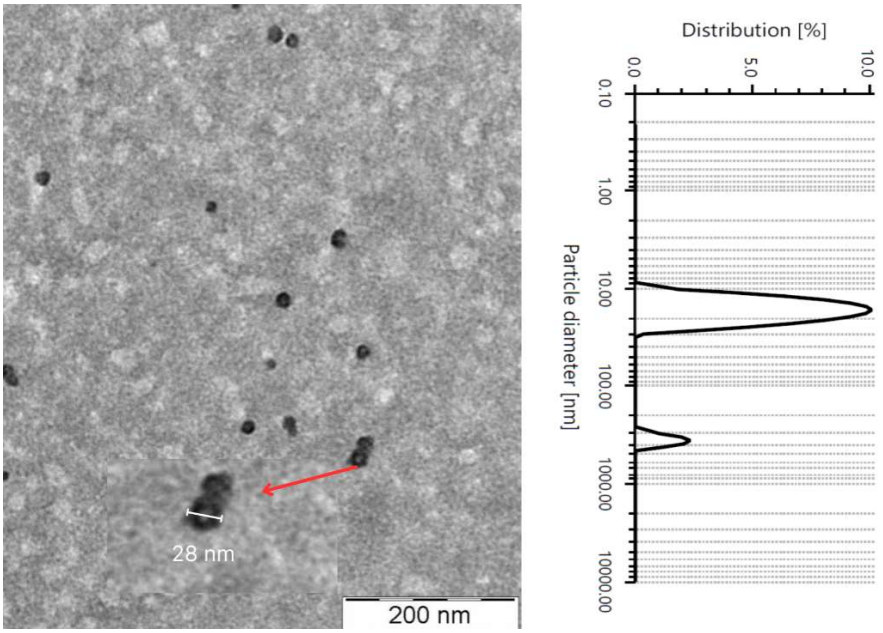

Figure 1: Particle size obtained by transmission electronic microscope (TEM) (left) and dynamic light scattering (DLS) (right)

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concentration of -3.08, corresponding to 0.0008% v/v.

The CMC signifies the lowest surfactant concentration required for self-assembly and encapsulation, transforming into a micelle. Below the CMC, surfactant molecules arrange themselves at the water surface, with the hydrophilic region orients inward, interacting with water molecules, while the hydrophobic region faces outward away from water.Once the surfactant concentration exceeds the CMC, self-assembly occurs, with the hydrophobic region positioning at the micelle's core and encapsulating the hydrophilic region.

Figure 2 demonstrates that the TPGS/Pol micelle exhibited a CMC of 0.0008% w/v. This finding aligns with previous studies, which reported CMC values for TPGS/Pol micelles at 0.0013% w/v (15)and 0.0015% w/v (14). Comparatively, the CMC value for TPGS micelles is 0.00052% w/v, while for Pol micelles it is 0.0575% w/v(25). Therefore, the combination of these two polymers yielded an intermediate value. Moreover, TPGS/Pol micelle exhibited a CMC value that leaned toward the CMC value of pure TPGS micelles, attributing to the greaterproportions of TGPSin the optimized formulation(14,22,25).

The evaluation of CMC holds crucial significance in nanoparticle studies, particularly for micelles. This is attributed to the potential disassembly of micelles in body fluids due to the extreme dilution below its CMC, which eventually affected the intended function of delivering drugs to targeted sites. Hence, micelles with lower CMC values could facilitate the effective drug transport owing to the heightened survivability and stability in body fluids.

In-vitro drug release study

Luteolin released without micelles exhibited a rapid release profile, reaching 100% within less than 4 hours. Conversely, a distinct pattern emerged in the release of luteolin encapsulated within micelles. A rapid initial release was demonstrated in the first 10 hours, followed by a steady increase in the release of luteolin over time in both

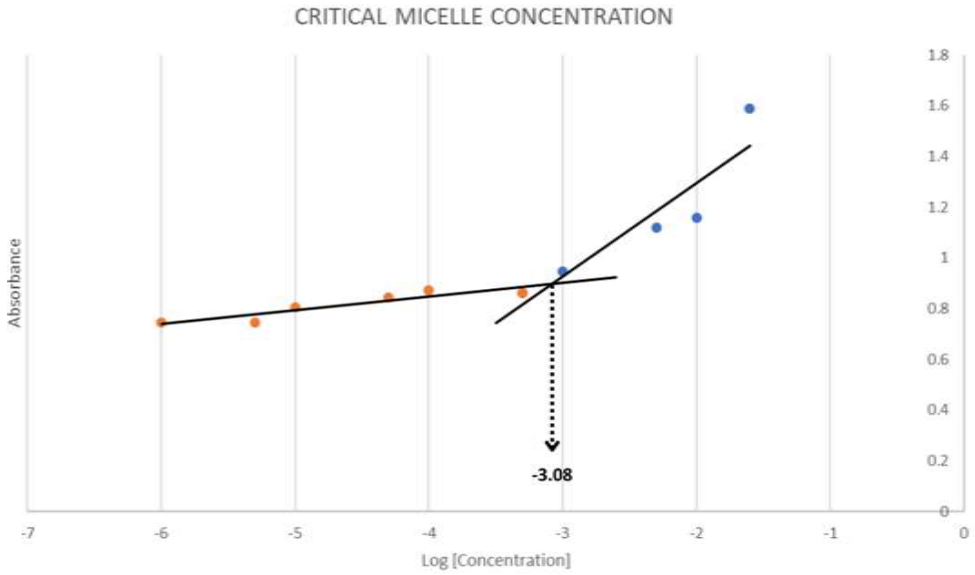

Figure 2: Critical micelle concentration determination by measuring the absorbance of KI/I_2 in diluted optimized blank micelle

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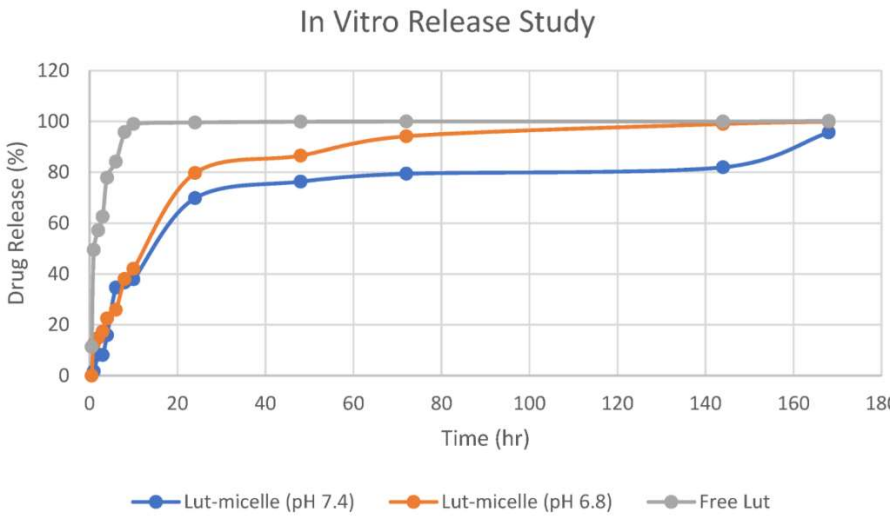

Figure 3: Release profile of free luteolin (grey), luteolin-loaded micelle in pH 7.4 (blue) and luteolin-loaded micelle in pH 6.8 (orange)

media. Remarkably, a continuous steady increase was observed for up to 7 days at physiological pH (pH 7.4). However, pH 6.8 resulted in a greater luteolin release compared to pH 7.4, possibly attributed to luteolin's increased solubility in lower pH environments due to partitioning effects(17). This information suggests that luteolin has the capability to be released liberally in slightly acidic tumour cells while exhibiting prolonged release in body fluids with physiological pH.

Figure 3 shows that free luteolinexhibited a more rapid release compared to luteolin-loaded micelles, with a continuous release observed for up to 7 days. This resultis consistent with prior research, where luteolin without micelles exhibited a burst-like release, whereas loading luteolin into micelles resulted in a sustained release(26–28). The sustained release behavior may result from the hydrolysis or degradation of themicelle, along with the polymer erosion and swelling, facilitating the luteolin to diffuse from micelle into the release medium.

Conclusion

The development and optimization of luteolin-loaded micelles using TPGS and Pol has been successfully achieved. The optimized micelle configuration was attained with TPGS: Pol ratio of 3:1 and a drug: polymer ratio of 1:5. This configuration exhibited remarkable characteristics, including a particle size below 40 nm, an EE of 90% and a CMC of 0.0008% w/v. Additionally, the micelledemonstrated a significantly enhanced solubility of luteolin, emphasizing its potential as a carrier for hydrophobic drugs. Furthermore, the sustained release behaviour of luteolin was demonstrated in the optimized micelle, showcasing controlled release over an extended period. Overall, this research lays a solid foundation for the utilization of TPGS/Pol micelles in drug delivery, particularly for hydrophobic compounds like luteolin, with potential implications for enhancing therapeutic efficacy and addressing solubility challenges in cancer treatment.

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Effects of Electronic Cigarette Use Amongst Cigarette Smokers In Klang Valley, Malaysia

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Abstract

Electronic cigarette (e-cigarette) is a battery powered device which imitates the feel and experiences of smoking a conventional cigarette. Recently, Malaysian government has banned the selling of e-cigarette and the nicotine-containing e-liquid. The decision may be influenced by studies from overseas. However, in-depth studies on the effectiveness of e-cigarette are needed especially in Malaysian setting. Thus, this study aimed to analyze the cost-effectiveness of using e-cigarette, the reduction in pack-year after using e-cigarette, % of users who completely changed from conventional cigarette to e-cigarette and to assess the general lung function of e-cigarette users using peak flow analysis in e-cigarette users in Klang Valley, Malaysia. This study was conducted at an e-cigarette stall in Kuala Lumpur Downtown Night Market, Cheras, Kuala Lumpur, Malaysia. Questionnaires were given to 73 e-cigarette users who met both inclusion and exclusion criteria. After answering the questionnaires, a peak flow meter test was carried out. The peak flow meter test was also carried out on conventional cigarette smokers for comparison. Findings from this study suggested that e-cigarette is a cost-effective device which showed a significant reduction of average monthly expenditure. In addition, results also showed that e-cigarette helped in the reduction of pack-year. The peak flow readings of the e-cigarette users are also significantly higher compared to the conventional cigarette smokers suggesting better general lung function in those who uses e-cigarette. In conclusion, e-cigarettes could serve as a viable alternative to nicotine replacement therapy (NRT) for traditional cigarette smokers seeking to quit. Nonetheless, it is imperative to conduct long-term studies on the potential side effects of e-cigarettes to thoroughly evaluate their benefits against potential risks.

Keywords: Electronic Cigarette, E-Cigarette, Pack-Year Reduction, Cost-Effectiveness, Peak Flow Analysis, Conventional Cigarette, Nicotine Replacement Therapy

Introduction

Breaking the habit of cigarette smoking poses significant challenges, underscoring the need for enhanced smoking cessation methods. E-cigarette have emerged as a potential substitute for traditional cigarette smoking. By replicating certain smoking behaviors, e-cigarettes offer a means for smokers to abstain during cessation efforts or reduce their cigarette intake. However, research into the long-term efficacy and safety of e-cigarettes for smoking cessation or reduction remains unclear [1].

The idea of using nicotine to reduce cigarette cravings was evidenced from at least in the 1940s as researchers have worked on the effects of hypodermic nicotine delivery test [2]. Following that, nicotine replacement therapy (NRT) was developed commercially in 1960. In Switzerland during 1978, the original nicotine gum was later followed by transdermal patches, nicotine nasal sprays, nicotine inhalers and nicotine microtabs [3]. NRT acts as an effective and cost-effective treatment for tobacco dependence [19]. In addition, according to United States of America (US) Food and

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Drug Administration [4], no tobacco products have been scientifically proven to reduce risk of tobacco-related disease, improve safety or cause less harm than other tobacco products.

According to the World Health Organization [5], the e-cigarette is one popular type of electronic nicotine delivery system (ENDS), a battery powered device that looks like a cigarette but does not involve smoke and enables users to inhale vaporized nicotine. Meanwhile, e-cigarette is also defined as an operated device that is typically made to look and perform like regular cigarette [4]. E-cigarette contains an inhalation activated mechanism that heats liquid from a cartridge composed of humectants and nicotine; although non-nicotine e-cigarette is also available [6]. E-cigarette users also known as “vapers” inhale the resulting vapor [4]. There are also many other terms, names, classifications, and definitions that have been used to describe e-cigarette. E-cigarette is also defined as a new type of device that delivers vaporized nicotine without the tobacco combustion or smoke of regular cigarette [7]. E-cigarettes are widely advertised as technologically advanced and healthier alternative to tobacco cigarette using youth-relevant appeals such as celebrity endorsement, trends, fashionable imaging and fruit, candy, and alcohol flavors [8].

The e-cigarette market has experienced rapid expansion, with vaping becoming increasingly popular among smokers in Malaysia and worldwide. The technology employed in e-cigarettes has garnered attention and has the potential to reshape public perceptions regarding smoking habits. Previous study noted that there is considerable interest in these devices and their potential impact on public health [9]. The benefits and risks of e-cigarettes are heavily influenced by public perceptions and usage patterns. It was suggested that the effectiveness of e-cigarettes in smoking cessation or reduction hinges on their ability to satisfy users and alleviate withdrawal symptoms [9]. However, research on the overall effects of e-cigarettes and their efficacy as cessation aids is still in its early stages. It was also emphasized the need for further research to gather comprehensive data on the advantages and disadvantages of e-cigarettes for public health [9].

Currently in Malaysia, e-cigarettes have emerged as a popular alternative for smokers looking to quit traditional cigarette smoking [10]. The adoption of e-cigarettes has surged, reflecting a growing trend. Recently, the Malaysian government implemented a ban on the sale of e-cigarettes and nicotine-containing e-liquids, possibly influenced by findings from international studies. However, there remains a need for comprehensive research into the efficacy of e-cigarettes, particularly within the Malaysian context. Thus, this study aims to evaluate the cost-effectiveness of e-cigarette use, the reduction in pack-years post-e-cigarette adoption, the percentage of users who transition completely from conventional cigarettes to e-cigarettes, and the general lung function of e-cigarette users using peak flow analysis in the Klang Valley, Malaysia. The findings of this study are expected to provide valuable insights for policy makers in formulating regulations governing the sales and usage of e-cigarettes in Malaysia.

Materials and Methods

Sampling Size

The sample size of 73 e-cigarette users was obtained using an online calculator to generate the proportion sample size from www.openepi.com. Confidence level of 95% and prevalence of 5% were used in this study. A population of roughly 7.5 million was taken from a geographical dictionary or directory website in conjunction with atlas (www.world-gazetteer.com).

Study site

The study was conducted at an e-cigarette stall in Kuala Lumpur Downtown

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Night Market, Bandar Tun Razak, Cheras, Kuala Lumpur for a period of one month. The location was chosen as the study site because it is one of the popular spots for e-cigarette users in Klang Valley. Respondents were targeted during the weekends which were on Friday, Saturday and Sunday from 10 pm to 2 am. Figure 2.1 shows the study site at Kuala Lumpur Downtown Night Market.

Inclusion and Exclusion criteria

E-cigarette users were included in the study if they live in Klang Valley during when this study was done, 18 years old and above and currently using e-cigarette. The e-cigarette users were excluded from this study if they had been using e-cigarette for less than 3 months, they were never a conventional cigarette smoker before and if they have asthma or any other obstructive pulmonary diseases.

Questionnaire

The tool used for the data collection in this study was validated questionnaires. The questionnaires were adopted and adapted from previous studies [11,12]. The questionnaires were used to find out on the cost-effectiveness of using e-cigarette, the reduction in pack-year after using e-cigarette, % of users who completely changed from

Figure 2.1: An e-cigarette stall at Kuala Lumpur Downtown Night Market, Cheras, Kuala Lumpur

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conventional cigarette to e-cigarette and to assess the general lung function of e-cigarette users using peak flow analysis in e-cigarette users. A peak flow test was conducted on both e-cigarette users and conventional cigarette smokers. Three readings were obtained for each of the respondents in order to get the average peak flow readings.

Data Analysis

Data was analyzed using Statistical Package for Social Sciences (SPSS) software version 20.0 for Windows. Paired sample T-test was used to measure whether means from subjects test group vary over two test conditions. Chi-square test was used to measure the strength of association between the variables. One way ANOVA was used to compare the sample means for a number of groups, multiple comparison methods for pairs of means and tests for the equality of the variances of the groups. Data were then tabulated and presented in tables and graphs using Microsoft Excel 2007.

Results and Discussion

DemographicData

Figure 3.1 shows the percentage of e-cigarette users by age group. Majority of the e-cigarette users were from age group of 31 to 35 years old with 31.5% (n=23), followed by the age group of 18 to 25 years old with 27.4% (n=20) and the age group of 26 to 30 years old with 24.7% (n=18). E-cigarette users in the age group of 36 to 40 years old and 41 to 45 years old shared the same percentage of 6.8% (n=5). The percentage of e-cigarette users by the age group of 46 to 50 years old and 56 to 60 years old showed the least percentage with only 1.4% (n=1) each.

Figure 3.2 shows the percentage of e-cigarette users by gender. All e-cigarette users were male 100% (n=73).

Figure 3.3 shows the percentage of e-cigarette users by class of income. Majority of the e-cigarette users were from the middle class of income which was between RM1000 to RM4000 with 75.3% (n=55), followed by the e-cigarette users from the class of income less than RM1000 with 16.4% (n=12). The percentage of e-cigarette users by the class of income more than RM 4000 showed the least percentage with 8.2% (n=6).

Cigarette Usage

Average monthly expenditure before using e-cigarette was obtained based on the cost of pack per day. Average monthly expenditure after using e-cigarette was obtained based on the cost of e-cigarette liquid and e-cigarette maintenance inclusive with conventional cigarette that the users still

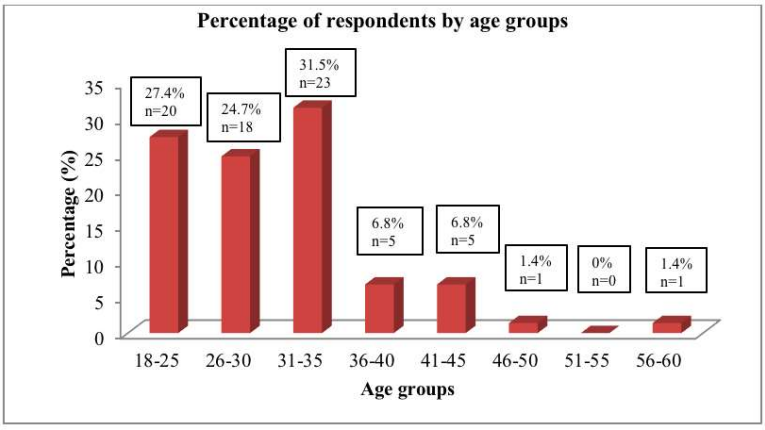

Figure 3.1: Bar chart of percentage of e-cigarette users by age groups

Electronic Cigarette Use Amongst Cigarette Smokers

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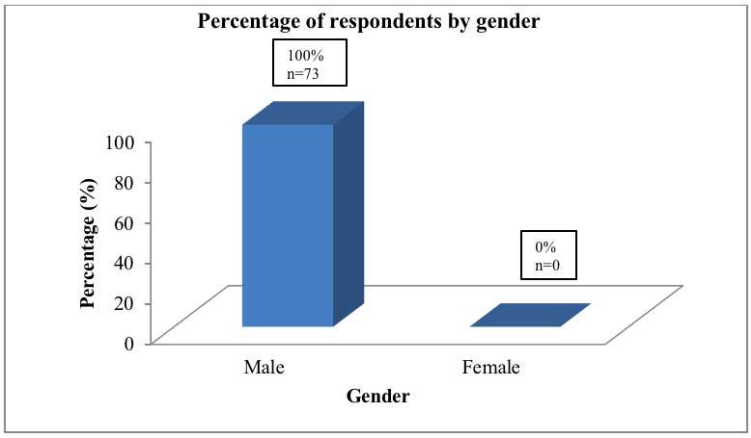

Figure 3.2: Bar chart of percentage of e-cigarette users by gender

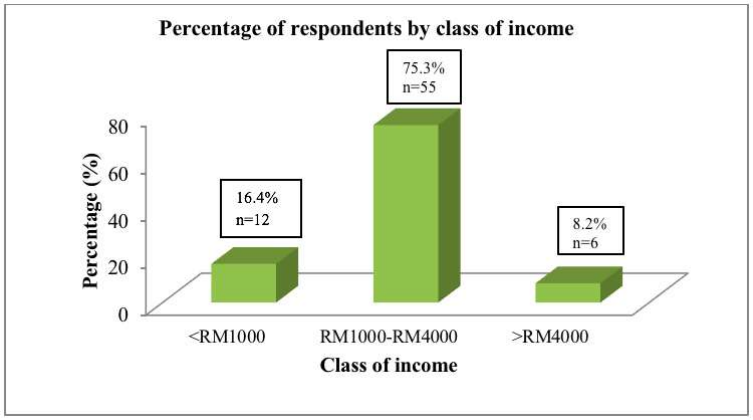

Figure 3.3: Bar chart of percentage of e-cigarette users by class of income

buy. Comparison between the average monthly expenditure before and after e-cigarette usage was done to evaluate the difference in term of cost on conventional cigarette and e-cigarette. Table 3.1 shows the mean of average monthly expenditure before and after e-cigarette usage. The mean for average monthly expenditure before e-cigarette usage was RM339.59 (±14.490) while the mean for average monthly expenditure after e-cigarette usage was RM117.67 (±4.531). The difference in the average monthly expenditure before and after e-cigarette usage was RM221.92 (±9.959). A paired-sample t-test was performed to compare the average monthly expenditure before e-cigarette usage with that of after e-cigarette usage. It was found that there is a significant difference in the mean of average monthly expenditure before e-cigarette usage when compared to that of the mean of average monthly expenditure after e-cigarette usage with ($p<0.05$). These results suggest that smokers spent a significant amount of money for conventional cigarette and that if the conventional cigarette smokers changed from conventional cigarette to e-cigarette, the conventional cigarette smokers could save a significant amount of money.

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Reduction in pack-year: Reduction of pack-year before and after e-cigarette usage

Table 3.2 shows the mean of pack-year before and after e-cigarette usage. The mean of pack-year before e-cigarette usage was 13.49 (±1.037) while the mean of pack-year after e-cigarette usage was 1.67 (±0.560). A paired sample t-test was performed to compare the mean of pack-year before e-cigarette usage with that of the mean of pack-year after e-cigarette usage. It was found that there is a significant difference in the mean of pack-year before e-cigarette usage when compared to that of the mean of pack-year after e-cigarette usage with ($p<0.05$). These results indicate that e-cigarette helps in the withdrawal of conventional cigarette consumption.

Association between classification of e-cigarette users based on their previous conventional cigarette pack-year and the tendency to completely change from conventional cigarette to e-cigarette

Conventional cigarette withdrawal is a problem associated with nicotine replacement therapy; thus, this association was made to further see the effect of e-cigarette on the classification of e-cigarette users based on their previous conventional cigarette pack-year towards the tendency to completely change from conventional cigarette to e-cigarette. In order to know whether there was an association between the classification of e-cigarette users as shown in Figure 3.4 and the tendency to completely change from conventional cigarette to e-cigarette, a Chi-square test was performed. Table 3.3 shows the percentage of respondents who completely changed to e-cigarette based on the classification of e-cigarette users. The e-cigarette users based on their previous conventional cigarette pack-year were classified into three; light, moderate and heavy smokerwhich was classified based on their previous pack-year of less than 15, 16 to 24 and more than 24 respectively. It was found that the light smoker had the highest percentage to completely change from conventional cigarette to e-cigarette with 58.9% (n=43), followed by the heavy smoker which completely change from conventional cigarette to e-cigarette with 8.2% (n=6). The lowest percentage to completely change from conventional cigarette to e-cigarette was the moderate smoker with 5.5% (n=4). However, there is no significant association between the classification of e-cigarette users and the tendency to completely change from conventional cigarette to e-cigarette. These results suggest that the tendency to completely change from conventional cigarette to e-cigarette was not associated with the classification of e-cigarette users.

Table 3.1: Mean of average monthly expenditure before andafter e-cigarette usage

Parameters	Before using electronic cigarette (RM)	After using electronic cigarette (RM)	(*p* value)
	Mean (±SEM)		
Average monthly expenditure	339.59 (±14.490)	117.67 (±4.531)	0.012[a]

Table 3.2: Mean of pack-year before and after e-cigarette usage

Parameters	Before using electronic cigarette	After using electronic cigarette	(*p* value)
	Mean (±SEM)		
Mean of pack-year	13.49 (±1.037)	1.67 (±0.560)	0.048[a]

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Peak flow reading (PEFR) between e-cigarette users and conventional cigarette smokers

In this study, the same number of conventional cigarette smokers were also recruited so that the peak flow reading of the conventional cigarette smokers could be compared with that of the peak flow reading of the e-cigarette users. In this present study too, e-cigarette users were further divided into two groups based on whether the users used the e-cigarette alone or whether the users used the e-cigarette in addition to conventional cigarette as described in Figure 3.5.

Figure 3.6 shows the general peak flow readings and frequency between conventional cigarette smokers and the e-cigarette users (e-cigarette users alone and e-cigarette users who also smoke conventional cigarette alternately or occasionally). Generally, the peak flow readings of the conventional cigarette smokers were less than 400 L/min and the peak flow readings of the e-cigarette users (e-cigarette users alone and e-cigarette users who also smoke conventional cigarette alternately or occasionally) were more than 500 L/min Figure 3.7.

Table 3.4 shows multiple comparisons for peak flow meter readings between the three groups which were e-cigarette users who used e-cigarette alone, e-cigarette users who used e-cigarette in

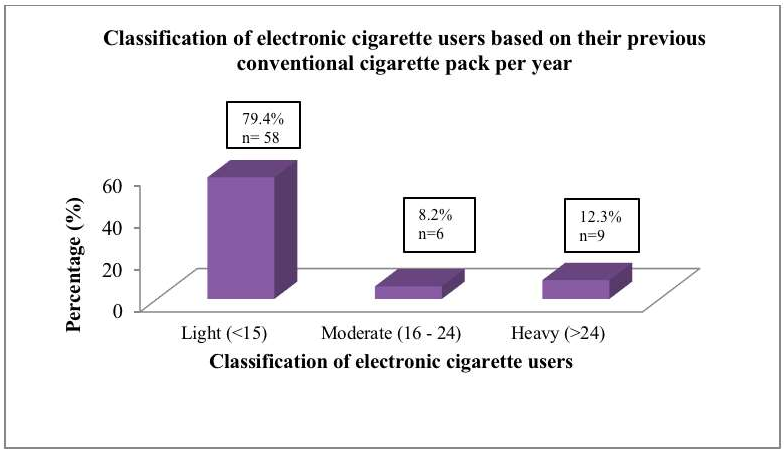

Figure 3.4: Bar chart of percentage and classification of e-cigarette users based on their previous conventional cigarette pack-year. Light smoker was a smoker with previous pack-year less than 15. Moderate smoker was a smoker with previous pack-year of 16 to 24. Heavy smoker was a smoker with previous pack-year of more than 24. The classification of the type of smoker based on pack-year was adapted from previous study [18]

Table 3.3: Percentage of respondents who completely changed from conventional cigarette to e-cigarette based on the classification of e-cigarette users (which was based on their previous conventional cigarette pack-year)

Type of electronic cigarette users based on their previous conventional cigarette pack per year	Completely change to electronic cigarette %		(*p* value)
	Yes	No	
Light (≤15)	58.9	20.5	
Moderate (16-24)	5.5	2.7	0.846[a]
Heavy (≥24)	8.2	4.2	

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addition to the conventional cigarette occasionally or alternately and conventional cigarette smokers. The means for e-cigarette alone, e-cigarette users who used e-cigarette in addition to the conventional cigarette occasionally or alternately and conventional cigarette smokers were 506.23 (±9.98), 495.00 (±14.40) and 393.29 (±2.63) respectively. A Tukey's post hoc test was done to further see the level of significance between the three groups. From the findings, it was found that there are significant differences in the mean peak flow readings of the e-cigarette users who used e-cigarette alone and e-cigarette users who used e-cigarette in addition to the conventional cigarette occasionally or alternately to that of the mean of peak flow readings of the conventional cigarette smokers ($p<0.001$; One way ANOVA with Tukey's post hoc test).

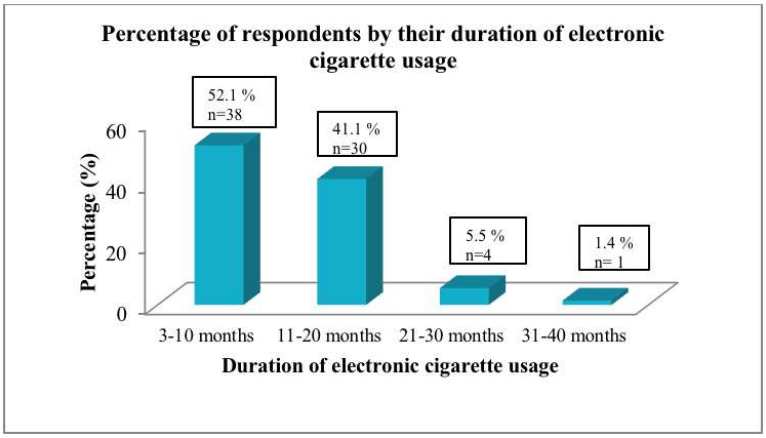

Figure 3.5: Bar chart of percentage of respondents by duration of e-cigarette usage

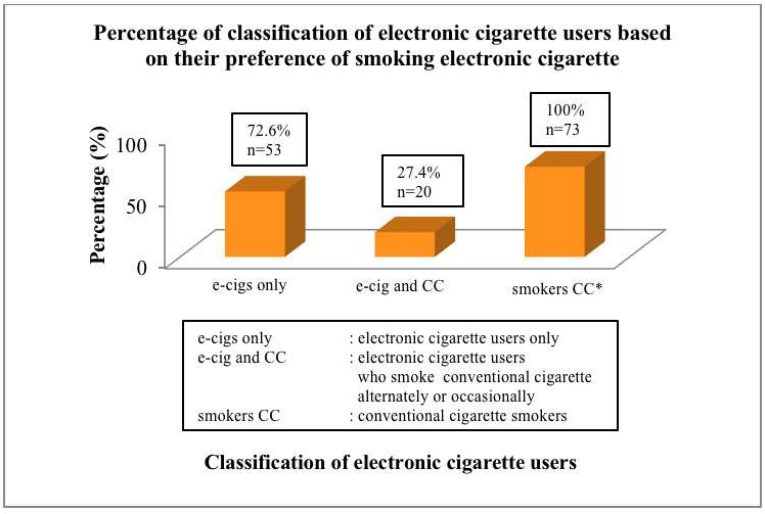

Figure 3.6: Bar chart of percentage of classification of e-cigarette users based on their preference of smoking e-cigarette. *Note that the smoker CC were sampled separately (n=73) to obtain their peak flow readings for 3.2.3

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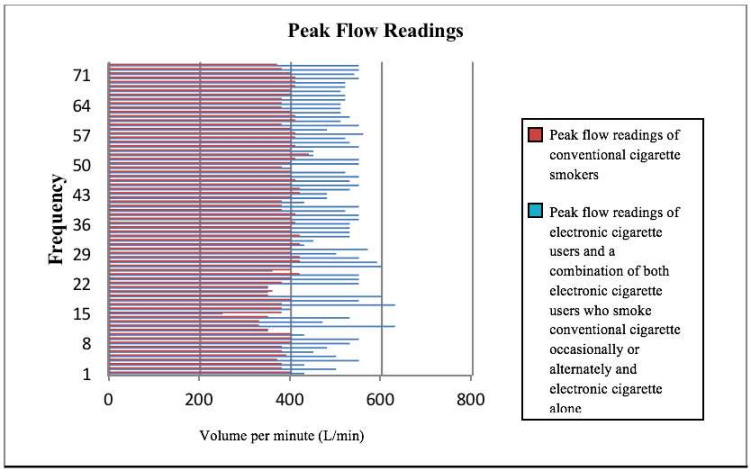

Figure 3.7: Peak flow readings between the conventional cigarette smokers and the e-cigarette users (e-cigarette users alone and e-cigarette users who also smoke conventional cigarette alternately or occasionally).

Table 3.4: Multiple comparisons for peak flow readings between three groups (which was based on their preferences of smoking e-cigarette as shown in Figure 3.6)

Dependent variables	(I) Groups	(J) Groups	(*p* value)
	Electronic cigarette users who used electronic cigarette alone (Mean = 506.23±9.98)	E-cigarette users who used e-cigarette in addition to the conventional cigarette	0.692
		Conventional cigarette smokers	0.000[d]
	Electronic cigarette users who used electronic cigarette in addition to the conventional cigarette (Mean = 495.00±14.40)	E-cigarette users who used electronic cigarette alone	0.692
		Conventional cigarette smokers	0.000[d]
	Conventional cigarette smokers (Mean = 393.29±2.63)	Electronic cigarette users who used electronic cigarette in addition to the conventional cigarette	0.000[d]
		Electronic cigarette users who used electronic cigarette alone	0.000[d]

[d]One-way ANOVA with Tukey's Post Hoc Test

Discussion

Cost effective: Difference in average monthly expenditure before and after e-cigarette usage

It has been a major issue for smokers at least in Malaysia that the average monthly expenditure for conventional cigarette is ever increasing. In 2007, excise tax duty was increased by 25%. As of 2010, tax constitutes about 54% of the retail price of popular brand cigarettes and these retail prices must get prior approval from the government. Cigarettes were set at a

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minimum price of RM15.70 per pack [13]. In addition, government is battling to reduce death due to smoking with increment in cigarette price. This has been a challenge for Malaysian smokers to continue smoking. However, cigarette addiction is tough to break thus some Malaysian smokers had opted for e-cigarette as an alternative. Concerns had been raised whether they use e-cigarette to cease smoking or to reduce average monthly expenditure. In the present study, the average monthly expenditure for smoking e-cigarette was evaluated and compared with that of the average expenditure before the respondents switched to e-cigarette. Based on the present study, it was found that there is a significant reduction in the average of monthly expenditure after the respondents opted to use e-cigarette.

The price of conventional cigarette in Malaysia is considered high compared to other countries. The price of e-cigarette is considerably cheaper as some e-liquid is made locally. When e-cigarette users slowly shifting from conventional cigarette, the e-cigarette users slowly saving some amount of money. One of the criteria looked upon in quit smoking is the cost. This is supported by a study done in US, daily users spent U$33 per month for e-cigarettes which is much cheaper than smoking one pack per day incurring the cost of U$150 to U$200 per month in respondents countries [12]. In comparison to the respondents of this study, average monthly expenditure before using e-cigarette was RM300 (U$94) to RM350 (U$110) and this amount decreased to RM100 (U$31) to RM150 (U$47) after the usage of e-cigarette taking the latest currency exchange rate at U$1 = RM3.18. The study shared that with a significant reduction in the average monthly expenditure, the e-cigarette may be a competitive alternative to conventional cigarette in an attempt to reduce the cost. However, no economic analyses were identified specifically addressing the cost effectiveness of nicotine replacement therapy in comparison with e-cigarette [14].

Reduction in pack-year: Reduction of pack-year before and after e-cigarette usage

Findings from this study show significant reduction of pack-year before and after using e-cigarette. Pack-year is calculated based on the sticks of the cigarettes times the years of smoking and divided by 20 sticks per pack. In the calculation, reduction in stick per day helped in reducing the pack-year. A study done on the effectiveness and tolerability of e-cigarette in real life in a 24-month prospective observational study showed that there was more than 50% reduction in cigarette smoking observed in 27.5% of participants, with a substantial reduction from 24 to 4 cigarettes per day [1]. In comparison with other nicotine replacement therapy, it was supported that nicotine replacement therapy does help in reduction of cigarette consumption. A study was done using nicotine replacement therapy in reducing cigarette consumption. The results from the study stated that, after one year, nicotine replacement therapy was effective in maintaining reduced consumption of e-cigarette by more than 50% of baseline level than placebo with 16 to 19% effectively [15]. The use of nicotine replacement therapy for smoking reduction and temporary abstinence was found to be positively associated with attempts to quit smoking and with abstinence at 6-month follow-up. Use of nicotine replacement therapy for smoking reduction and temporary abstinence was associated with a small reduction in daily cigarette consumption [16].

E-cigarette users experience a reduction in pack-year once they switched from conventional cigarette to e-cigarette. Not only e-cigarette reduced the cost, shifting to e-cigarette may be beneficial as conventional cigarette is the main cause of lung cancer once being exposed to the carcinogenic substances such as tar and carbon monoxide. Even though there is reduction in cigarette consumption, a proper study is still crucial to thoroughly evaluate if there is any reduction in nicotine intake.

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Association between classification of ecigarette users based on their previous conventional cigarette pack-year and the tendency to change from conventional cigarette to e-cigarette

Findings from this study show that there is no significant association between classification of e-cigarette users based on their previous conventional cigarette pack-year and the tendency to change from conventional cigarette to e-cigarette. The e-cigarette users based on their previous conventional cigarette pack-year were classified into three. In this study, smokers with the lowest pack per year tend to change into e-cigarette completely. E-cigarette helps the smokers in competing with withdrawal symptoms of cigarette. This is supported by a survey with responses of 216 first time buyers of e-cigarette, about two thirds of participants 66.8% reduced cigarette consumption, almost half of them 48.8% were temporarily smoke free and 31% of users reported being completely smoke free within six months after starting to use e-cigarette. Most of these former conventional cigarette smokers 56.7% continue to use e-cigarette but only about one third of them have stopped using nicotine containing products [12]. In addition, e-cigarette reduces the desire to smoke and nicotine withdrawal symptoms after 20 mins of its usage [17].

Limitation of Study

Several limitations were identified during the execution of this study. Firstly, the sample size was generally small, which may not fully represent all e-cigarette users in Klang Valley. Additionally, the survey was conducted at a single location, potentially introducing site bias and influencing the outcomes. However, it's noteworthy that the chosen study site, Kuala Lumpur Downtown Night Market, Cheras, Kuala Lumpur, serves as a primary hub for e-cigarette users to access device maintenance and liquid. Future studies should consider conducting surveys at multiple locations to enhance the reliability and accuracy of the findings. Regarding peak flow readings, many were obtained only once from each e-cigarette user for convenience, and some users declined multiple readings, potentially introducing bias and impacting the results. To address this in future research, a prospective study could be implemented to closely monitor each respondent's cigarette consumption and provide more robust data on reduction outcomes.

Conclusion

Findings from this study suggested that e-cigarette is a cost-effective alternative to conventional cigarette. Besides, e-cigarette reduces cigarette consumption which leads to decrease in pack-year amongst users and better peak flow readings which may indicate better lung function. Therefore, the results of this study imply that e-cigarettes could be a promising approach for smoking cessation and merit further investigation and research design.

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Knowledge and Perceived Benefits of Electronic Cigarette Among Users In Klang Valley, Malaysia

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Abstract

Electronic cigarette (e-cigarette) is a battery powered device which imitates the feel and experiences of smoking a conventional cigarette. This device has gained considerable attention since their inception into the European and American markets in the early 2000s and have become increasingly common in many countries, including Malaysia. As of 2020, 33.7% of Malaysians reported ever using ECs and 5.4% used ECs on daily basis.The aim of this study was to gauge the level of knowledge and perceived benefits of e-cigarette users in Klang Valley, Malaysia. This study was conducted at an e-cigarette stall in Kuala Lumpur Downtown Night Market, Cheras, Kuala Lumpur. Questionnaires were given to 73 respondents that met both the inclusion and exclusion criteria. Out of 73 participants, 54.8% were regarded as having a medium level of knowledge while the rest were under high level of knowledge. Findings from this study also showed that there are no significant associations between duration of e-cigarette usage and class of income with the levels of knowledge of the e-cigarette users on this device. High percentage of respondents strongly agreed that e-cigarette had positive perceived benefits which outweigh the risks. As a conclusion, this battery powered device may be considered as an alternative to nicotine replacement therapy (NRT) available in an attempt to quit smoking.

Keywords: Electronic Cigarette, E-Cigarette, Perceived Benefits, Knowledge, E-Cig, Smoking Cessation, Nicotine Replacement Therapy, Malaysia

Introduction

In recent years, the use of electronic cigarettes (e-cigarettes) has garnered significant attention globally, presenting a complex landscape of public health challenges and opportunities. Malaysia, particularly the bustling urban region of Klang Valley, has not been immune to this trend. As the prevalence of e-cigarette use rises, it becomes imperative to investigate the knowledge and perceived benefits of these devices among users within this dynamic Malaysian setting.

E-cigarettes, also known as electronic nicotine delivery systems (ENDS), are battery-operated devices that vaporize a liquid solution containing nicotine, flavorings, and other chemicals, which users inhale. Initially marketed as a safer alternative to traditional tobacco smoking, e-cigarettes have since evolved into a multifaceted phenomenon with diverse implications for public health [1]. Despite ongoing debates surrounding their efficacy as smoking cessation aids and potential long-term health effects, e-cigarettes have gained popularity among individuals of various demographics, including youth and adults [2].

In the context of Malaysia, where tobacco control measures have been implemented rigorously, understanding the knowledge and perceived benefits of e-cigarettes among users is of paramount importance. Malaysia's regulatory framework includes prohibitions on smoking in public places, restrictions on tobacco advertising, and taxation on tobacco products, which may influence patterns of e-cigarette use and perceptions of harm reduction [3]. However, limited research has been conducted to

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elucidate the factors driving e-cigarette use and the extent to which users are aware of their potential benefits and risks within this sociocultural context.

Moreover, the Klang Valley region, encompassing Kuala Lumpur and its surrounding urban areas, represents a diverse and dynamic microcosm of Malaysian society. Its cosmopolitan nature, coupled with socioeconomic disparities and cultural influences, may shape attitudes and behaviors related to e-cigarette use in unique ways. By examining the knowledge and perceived benefits of e-cigarettes among users in Klang Valley, insights can be gleaned to inform targeted interventions and policies aimed at mitigating potential harms and maximizing public health outcomes.

This study seeks to address this gap by conducting a comprehensive investigation into the knowledge and perceived benefits of e-cigarettes among users in Klang Valley, Malaysia. Through rigorous empirical research, we aim to elucidate the factors influencing e-cigarette use behaviors, assess users' understanding of the risks and benefits associated with these devices, and identify potential avenues for harm reduction strategies tailored to the Malaysian context.

Materials And Method

Sampling Size

The sample size of 73 e-cigarette users was obtained using an online calculator to generate the proportion sample size from www.openepi.com. Confidence level of 95% and prevalence of 5% were used in this study. A population of roughly 7.5 million was taken from a geographical dictionary or directory website in conjunction with atlas (www.world-gazetteer.com).

Study site

The study was conducted at an e-cigarette stall in Kuala Lumpur Downtown Night Market, Bandar Tun Razak, Cheras, Kuala Lumpur for a period of one month. The location was chosen as the study site because it is one of the popular spots for e-cigarette users in Klang Valley. Respondents were targeted during the weekends which were on Friday, Saturday and Sunday from 10pm to 2am.

Inclusion and Exclusion criteria

E-cigarette users were included in the study if they live in Klang Valley during when this study was done, 18 years old and above and currently using e-cigarette. The e-cigarette users were excluded from this study if they had been using e-cigarette for less than 3 months, they were never a conventional cigarette smoker before and if they have asthma or any other obstructive pulmonary diseases.

Questionnaire

The tool used for the data collection in this study was validated questionnaires. The questionnaires were adopted and adapted from previous studies [4,5]. The questionnaires were used to gauge the level of knowledge of users on e-cigarette and their perceived benefits of e-cigarette use as outlined in Tables 1 and 2.

Data Analysis

Descriptive analysis was presented in percentage (%) and association test was analysed using Chi-square Test using the Statistical Package for Social Sciences (SPSS) software version 20.0 for Windows.

Results and Discussion

Level of Knowledge on Electronic Cigarette

In this study, level of knowledge was categorized into three groups which were low level of knowledge with a score less than 2, moderate level of knowledge with a score of 2 to 5 and high level of knowledge with a score more than 5. A low level of knowledge was not shown in the results because no participant scored below 2. Most of the respondents in this study had a medium level

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Table 1: Questionnaire on knowledgeon electronic cigarette

Questionnaire on knowledge on electronic cigarette*:* *Soal selidik berkenaan pengetahuan tentang rokok elektronik :*		
Question/*Soalan*	Yes/*Ya*	No/*Tidak*
Do you know that the liquid of electronic cigarette contains nicotine? *Adakah anda tahu bahawa cecair rokok elektronik mengandungi nikotin?*		
Do you know that electronic cigarette contains the following :	Yes/*Ya*	No/*Tidak*
a. *Adakah anda tahu bahawa rokok elektronik mengandungi bahan berikut:* Propylene glycol/*Propylene glycol*		
b. Dietylene glycol/*Dietyleneglycol*		
c. Vegetable glycerin/*Glycerintumbuh-tumbuhan*		
Do you know that electronic cigarette can cause addiction? *Adakah anda tahu bahawa rokok elektronik boleh mengakibatkan ketagihan?*		
How would electronic cigarette be best described? *Bagaimana rokok elektronik tepat digambarkan?*		
a. Nicotine replacement therapy/*Terapipenggantinikotin* b. Harm reduction nicotine-delivery-system/ *Sistem penghantaran nikotin yang kurang berbahaya* c. Same with normal cigarette/*Sama sepertirokokbiasa*		
Do you know that in Malaysia, the SALE of nicotine containing liquid of electronic cigarettes is an offense under the Poison Act 1952 and the Control of Drugs and Cosmetics Regulations 1984? *Adakah anda tahu bahawa di Malaysia,* *Penjualan cecair rokok elektronik yang* *Mengandungi nikotin adalah menyalahi undang- undang di* *Bawah Akta Racun 1952 dan Peraturan peraturan Kawalan Dadah Kosmetik 1984?*		

of knowledge with 54.8% while 45.2% were regarded as having high level of knowledge on e-cigarette (Table 3).

Association between the Level of Knowledge on Electronic Cigarette and Class of Income

Table 4 shows the association between the levels of knowledge on e-cigarette and the class of income. The highest percentage for the moderate level of knowledge was from the class of income between RM1000 to RM4000 with 42.5% (n=31), followed by the class of income less than RM1000 with 8.2% (n=6). The lowest percentage for the moderate level of knowledge was from the class of income more than RM5000 with 4.1% (n=3). The highest percentage for the high level of knowledge was from the class of income between RM1000 to RM4000 with 32.9% (n=24), followed by income less than RM1000 with 8.2% (n=6). The lowest percentage for the high level of knowledge was from the class of income of more than RM5000 with 4.1% (n=3). A Chi-square test was performed to see the association between the levels of knowledge on e-cigarette and the class of income. It was found that there is no significant association between the levels of knowledge on e-cigarette and the class of income. These results suggest that the levels of knowledge of the e-cigarette users on e-cigarette are not affected by the class of income.

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Table 2: Questionnaire on perceived benefits on the use of electronic cigarette

Questionnaire on perceived benefits of electronic cigarette: *Soal selidik berkenaan faedah tanggapan rokok elektronik:*					
Question/*Soalan*	1	2	3	4	5
1. What noticeable changes have you experienced upon switching to electronic cigarette? *Apakah perubahan yang* *Dapat anda rasai apabila bertukar kepada rokok elektronik* a. Reduction in persistent cough. *Pengurangan dalam batuk berpanjangan.*					
b. Reduction in occurrence of shortness of breath. *Pengurangan dalam masalah sesak nafas.*					
c. Reduction in occurrence of sore throat. *Pengurangan dalam masalah sakit tekak.*					
d. Reduction in mouth dryness. *Pengurangan dalam masalah mulut kering.*					
e. Reduction in bad breath. *Pengurangan dalam masalah mulut berbau.*					
f. Reduction in craving for conventional cigarette. *Pengurangan dalam keinginan untuk merokok rokok biasa.*					
g. Increased in the general health. *Meningkatkan tahap kesihatan.*					
h. Increased in ability to perform exercise. *Meningkatkan keupayaan untuk melakukan senaman.*					
i. Increased in sense of taste. *Meningkatkan deria rasa.*					
j. Increased in sense of smell. *Meningkatkan deria bau.*					

Table 3: Level of knowledge of users on electronic cigarette

Knowledge (%)	
Moderate (2-5)	High (>5)
54.8	45.2

Association between the Level of Knowledge on Electronic Cigarette and Duration of Electronic Cigarette Usage

This association was done in order to assess the improvement of knowledge after using e-cigarette for a period of time. Table 5 shows the association between the levels of knowledge on e-cigarette and duration of e-cigarette usage. The highest percentage for the moderate level of knowledge was from the duration of e-cigarette usage between 3 to 10 months with 28.8% (n=21). The lowest percentage for the moderate level of knowledge was from the duration of e-cigarette usage between 31 to 40 months with 0.0% (n=0). The highest percentage for the high level of knowledge was from the duration of e-cigarette usage between 3 to 10 months with 23.3% (n=17). The lowest

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Table 4: Association between the levels of knowledge on electronic cigarette and class of income

Income	Knowledge (%)		(*p* value)
	Moderate (2-5)	High (>5)	
<RM1000	8.2	8.2	0.895[b]
RM1000 - RM4000	42.5	32.9	
>RM5000	4.1	4.1	

[b]*Chi-square test*

Table 5: Association between the levels of knowledge on electronic cigarette and duration of electronic cigarette usage

Months of electronic cigarette usage	Knowledge (%)		(*p* value)
	Moderate (2-5)	High (>5)	
3-10	28.8	23.3	0.593[b]
11-20	21.9	19.2	
21-30	4.1	1.4	
31-40	0.0	1.4	

[b]*Chi-square test*

percentage for the high level of knowledge was from duration of e-cigarette usage between 21 to 30 months and 31 to 40 months both with 1.4% (n=1). A Chi-square test was performed to see the association between the levels of knowledge on e-cigarette and duration of e-cigarette usage. It was found that there is no significant association between the levels of knowledge on e-cigarette and duration of e-cigarette usage. These results suggest that the levels of knowledge of the e-cigarette users on e-cigarette is not affected by the duration of e-cigarette usage.

Association between the Level of Knowledge on Electronic Cigarette and Age Group of Respondents

Table 6 shows the association between level of knowledge and respondents age. The highest percentage for the moderate level of knowledge was from respondents with age of 18 to 25 years old with 16.3% (n=11). The lowest percentage for the moderate level of knowledge was from respondents with age of 46 to 50 years old with 0.0% (n=0). The highest percentage for the high level of knowledge was from respondents with age of 26 to 30 years old with 17.4% (n=12). The lowest percentage for the high level of knowledge was from respondents with age of 46 to 60 years old with 0.0% (n=0). A Chi-square test was performed to see the association between levels of knowledge with age of respondents. It was found that there is no significant association between the levels of knowledge and age of respondents. These results suggest that the difference in age did not affect the levels of knowledge of e-cigarette users.

Perceived Benefits of Electronic Cigarette among Users

The questionnaire included ten narratives aimed at eliciting feedback from users regarding the perceived benefits of e-cigarettes. Table 7 shows the percentage of perceived benefits reported by respondents on a Likert scale. Respondents were asked

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Table 6: Association between levels of knowledge on electronic cigarette and age of respondents

Age	Knowledge (%)		(*p* value)
	Moderate (2-5)	High (>5)	
18-25	16.3	10.9	0.706[b]
26-30	12.3	17.4	
31-35	13.6	15.1	
36-40	4.1	2.8	
41-45	5.5	1.4	
46-50	0.0	0.0	
51-55	1.4	0.0	
56-60	1.4	0.0	

[b]*Chi-square test*

Table 7: Mean of percentage of perceived benefits claimed by the respondents

Perceived benefits	1 Strongly disagree (%)	2 Disagree (%)	3 Neutral (%)	4 Agree (%)	5 Strongly agree (%)
Reduction in persistent cough.	0.0	0.0	5.0	13.7	79.5
Reduction in occurrence of shortness of breath.	0.0	0.0	1.4	15.1	83.6
Reduction in occurrence of sore throat.	0.0	0.0	6.8	16.4	76.7
Reduction in mouth dryness.	1.4	1.4	5.5	16.4	75.3
Reduction in bad breath.	0.0	0.0	1.4	16.4	82.2
Reduction in craving for conventional cigarette.	1.4	1.4	13.7	50.7	32.9
Increase in general health.	0.0	0.0	6.8	39.7	53.4
Increase in ability to perform exercise.	0.0	2.7	5.5	21.9	69.9
Increase in sense of taste.	1.4	1.4	5.5	17.8	74.0
Increase in sense of smell.	1.4	1.4	4.1	20.5	72.6

to rate their agreement using the Likert scale, with options ranging from strongly agree (score 5) to strongly disagree (score 1). According to Table 7, for nine out of the ten perceived benefits narratives, over 50% of respondents strongly agreed (score 5). These included reductions in persistent cough, shortness of breath, sore throat, mouth dryness, bad breath, as well as improvements in general health, exercise performance, taste, and smell perception. However, the narrative concerning a reduction in craving for conventional cigarettes received the lowest percentage of strongly agree responses. Notably, none of the respondents strongly disagreed (score

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1) or disagreed (score 2) with five of the perceived benefits narratives, which included reductions in persistent cough, shortness of breath, sore throat, bad breath, and improvements in general health. Additionally, less than 15% of respondents provided neutral responses (score 3) for all ten perceived benefits narratives. In summary, the overall consensus among respondents suggests that transitioning to e-cigarettes has yielded positive outcomes.

Discussion

Findings from this study show that there is no significant association between levels of knowledge on e-cigarette with the class of income, duration of e-cigarette usage and age of respondents. Levels of knowledge do not rely on the increment of the income as well as duration of using e-cigarette and age of respondents. There are other contributing factors towards the level of knowledge. One of the factors is mass media. Mass media campaigns can raise awareness and change attitudes about the risk of using tobacco and benefits of quitting [6]. E-cigarette advertisements and related promotion activities are spreading to adolescents and internationally through the internet [7]. The spreading of the information indirectly contributes to the level of knowledge regarding e-cigarette.

Based on the results obtained, respondents from the age group of 18 to 35 years old had the highest percentage of knowledge. This may be due to their exposure towards e-cigarette on social media. In addition, e-cigarette is being published via bloggers and specific brand websites for this device. Thus, these phenomena actually targeting youngsters to gauge some information regarding this electronic nicotine delivery device system (ENDS). Examples of e-cigarette brands websites available are Vapor4Life, ProVape and MadVape. This is one of the factors that influenced the levels of knowledge of respondents based on age.

Besides, limited studies and published facts about e-cigarette also do affect the level of knowledge of respondents towards this device. The limited published research about the safety, composition, efficacy and public health impact of e-cigarette increases the concerns of public regarding this device [8]. Questions regarding regulations and ingredients of liquid nicotine can be answered well if the respondents are well aware of the composition and safety of the device. Knowledge on the ingredient and device itself can be obtained through exposure and awareness campaigns that will increase the public concerns regarding e-cigarette[9]. The results from this study and evidences obtained suggested that knowledge are associated with other contributing factors instead of class of income, duration of e-cigarette usage and age.

E-cigarette is an alternative to the nicotine replacement therapy (NRT) available. The only difference is e-cigarette is a battery powered nicotine delivery device. Examples of NRT are nicotine gum, nicotine patch and nicotine lozenge [10]. E-cigarette appears to help smokers transition to a less harmful replacement tool, thereby maintaining cigarette abstinent due to their perceived benefits compared to other nicotine replacement therapy [11].

Findings from this study showed that e-cigarette does have perceived benefits based on the response from the respondents. Respondents were asked to answer the perceived benefits narratives based on the likert scale of score 1 (strongly disagree) up to score 5 (strongly agree). High percentage of respondents strongly agreed that e-cigarette perceived benefits of reduction in persistent cough (79.5%). This is because of the composition in e-cigarette such as propylene glycol, diethylene glycol, ethylene glycol, nicotine and glycerin that do not give irritation to the consumers [12]. Respondents also perceived that e-cigarette does help in reduction of occurrence of shortness of breath (83.6%). In comparison to conventional cigarette that contains 4000 chemical constituents, consists of

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carcinogenic constituents such as tar which is nicotine free, dry, particulate mass of tobacco smoke that would accumulate and darken the lungs which in turn lead to shortness of breath. In addition, respondents also perceived that e-cigarette does help in reduction in craving for conventional cigarette (32.9%) [12].

Previous study revealed that 70% to 90% of e-cigarette consumers have less desire to smoke when using e-cigarette and this is what perceived by the respondents from this study [13]. This is also supported that e-cigarette can reduce desire to smoke and nicotine withdrawal symptoms 20 minutes after use [14]. High percentage of respondents also perceived that e-cigarette do help in reduction of occurrence of sore throat (76.7%), reduction in mouth dryness (75.3%), reduction in bad breath (82.2%), increased in general health (53.4%), increased in ability to perform exercise (69.9%), increased in sense of taste (74.0%) and increased in sense of smell (72.6%) significantly.

Results of this study regarding perceived benefits of e-cigarette suggested that this device has several benefits that outweigh the negative assumptions.

Despite the insights gained from this study, further research is warranted to deepen our understanding of e-cigarette usage and perceptions. Future studies could explore additional factors that may influence knowledge levels and perceptions among e-cigarette users, such as educational background, smoking history, and exposure to marketing messages. Additionally, longitudinal studies tracking changes in knowledge and perceptions over time could provide valuable insights into the evolving landscape of e-cigarette use. Moreover, qualitative research methods, such as interviews and focus groups, could offer nuanced perspectives on the motivations and experiences of e-cigarette users. By addressing these research gaps, future studies can contribute to a comprehensive understanding of e-cigarette usage patterns and inform evidence-based strategies for public health intervention and policy development.

Conclusion

In this study, the majority of respondents exhibited moderate knowledge (54.8%), while 45.2% demonstrated high knowledge levels. Associations between knowledge levels and income class, duration of e-cigarette usage, and age group were explored, revealing no significant relationships, suggesting that these factors do not influence knowledge levels among e-cigarette users. Additionally, respondents overwhelmingly agreed with perceived benefits of e-cigarettes, with positive outcomes such as reduced cough, shortness of breath, sore throat, and improved general health, indicating favorable perceptions of e-cigarette usage.

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Development of Biodegradable Tapioca Starch Films Incorporating Green Synthesized Zinc oxide Nanoparticles for Enhanced Preservation and Packaging of Sweet lime Segments: A Study of Their Physical and Antimicrobial Properties

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Abstract

Packaging has been on the environmental agenda for decades. It has been discussed and debated within the society as an environmental problem and the focus has been on the packaging material, including recycling options.In this research workgreen synthesized zinc oxide nanoparticle was performed using clove and cinnamon extract. Two types of tapioca starch based food packaging films (F1 and F2) were produced. In these F2 film had embedded zinc oxide nanoparticles in it whereas, F1 was used as control food packaging film without any nanoparticles. These films were used to wrap sweet lime segments and their advantages were tested via functional properties, characterization and quantitative assessments. The procedure was standardized, filmswere developed and physical properties were tested. Tapioca starch film F2 had the least moisture content (10.21%±0.25), swelling index (28.37%±0.14) & solubility (21.63%±0.42), was smooth, flexible and F1 film had the most transparency. F1 hadthe high values of moisture content (11.03%±0.78), swelling index (27.02%±0.35) & solubility (20.70%±0.74), was fine, flexible, and had better transparency.Film F2 also proved to have a significant antimicrobial activity. Thus, from the overall results tapioca starch film F2 was found to be the better option for food packaging applications when comparedtoF1 film.

Keywords: Zinc Oxide Nanoparticle, Tapioca Starch, Green Synthesis, Sweet Lime Segments

Introduction

Food packaging ensures the safety of food products, facilitates easy handling and transportation, and prevents chemical contamination, extending shelf life and offering added convenience to consumers. Food packaging has been made from a variety of materials, such as plastics, crystals, alloys, papers, and their complexes. The relevance of transferring dangerous elements from the packaging materials into the foods, majority, is of more worry as a result of customers' heightened health awareness. Most materials used for food

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packaging today are not biodegradable, leading to environmental problems (1). Regarding residual monomer and other plastics' stabilizers, plasticizers, and condensation-related components like bisphenol A, there have been certain health worries raised. To create materials for eco-friendly food packaging, a number of biofilms have been investigated. Because of their significant advantages over plastics, such as biodegradability, environmental friendly, low toxicity, and biocompatibility, the practice of biofilms as food packaging materials is starting to gain popularity on a globalscale (3). These substances provide outstanding cohesive film-forming properties as well as thin film layers of protection. Currently, materials for food packaging employ biofilms such starch, cellulose, and polylactic acid (PLA).

Polysaccharides, proteins, and aliphatic polyesters make up the majority of biofilms used as food packaging materials. These materials help preserve food quality and lengthen a product's shelf life. These packaging materials can shield food products from the external environment and stop the harm of desirable elements like flavour and texture thanks to their barrier qualities that regulate the exchange of gases, moisture, fragrance, and lipids. The use of innovative, high-performing, light weight, and environmentally friendly composite materials is made possible by the use of biofilms, which can replace conventional non-biodegradable plastic packaging materials. Because they are biodegradable and non-toxic, polysaccharides found in biofilms such chitosan, carboxymethyl cellulose, and starch may be employed to address ecological problems. In addition to these benefits, typical biofilms have several drawbacks, such as weak mechanical qualities and low water resistance. In order to increase the heat stable, mechanical, and gas retaining qualities while maintaining their biodegradability and low toxicity, nano biofilms are utilized (2). When included in biofilms, nanoparticles have a proportionately higher surface area than their microscale counterparts, which favours the interactions between the filler and matrix and the functionality of the resultant materials. In addition to serving as nano reinforcements, nanoparticles can serve a variety of purposes in polymers, including biosensing, enzyme immobilization, antibacterial activity, and others.

Due to its trivial, affordable, visible, adaptable, and simple-to-process qualities, plastic, a petroleum-based, diversified, and pervasive material, is frequently employed in food packaging. An estimated 20% rise in plastic consumption from current levels of 6% by 2050. Plastic garbage accumulates over time owing to extended degradation, harming terrestrial ecosystems and polluting marine ones (5). During abiotic and biotic breakdown, landfill plastics produce hazardous chemicals that contaminate soil and water. While the decomposition of plastic in water releases compounds like polystyrene, chlorinated plastics leach hazardous chemicals that harm ecosystems. Global warming is caused by methane and CO2 emissions produced by microbes that break down plastic. Plastic garbage exposes animals by ingestion and entanglement, which has negative effects.

Zinc oxide nanoparticles have a variety of shapes and effectively prevent the growth of a wide range of bacterial species (4). In ready-to-eat poultry meat, zinc oxide nanoparticles have been shown to have antibacterial activity against *Salmonella typhimurium* and *Staphylococcus aureus.* These zinc oxide-Nanoparticles may also have the ability to shield food against bacterial contamination (12). Studies have shown that compared to other metal oxides, zinc oxide nanoparticles are more effective against *Escherichiacoli, Staphylococcus aureus, Pseudomonas aeruginosa* species. Zinc oxide nanoparticles are more attractive for packaging applications than silver nanoparticles since they are less expensive and unharmful to both humans and animals. Additionally, due to the antibacterial properties exhibited by zinc oxide nanoparticles, they can generate a substantial amount of hydrogen peroxide upon exposure to UV irradiation. This leads to oxidative stress in bacterial cells. (4*).*

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Several natural materials can be used to create different types of biofilm packaging. The polysaccharide family has succeeded in creating fresh origin materials that may now be used in place of their nonbiodegradable petrochemical-based equivalents by adding hardness, viscosity, and gel-forming capacity, it has the ability to create and contribute to the production of a range of polysaccharide films (9). Because they are harmless, they may biodegrade and do not leave behind any damaging byproducts for the environment. Additionally, it possesses exceptional gas permeability qualities that increase the product's shelf life. Materials being explored for biodegradable packaging films include polysaccharides like starch, cellulose, and chitosan. These polysaccharides can create films that are effective at blocking the exchange of gases like oxygen and carbon dioxide. Tensile strength and elongation percentage, on the other hand, are crucial mechanical attributes because it depends on them to maintain the quality of the packed food (8). The three main sources of starch for commercial production are potatoes, corn, and wheat. One of the most prevalent and commonly utilized polymers in the packaging business is cellulose and its derivatives. A crucial polymer in the food sector is cellulose. It is the well-known polysaccharide that degrades naturally. Chitosan is the second most prevalent polysaccharide in the world after cellulose. Commercial supplies of these biofilms are currently in abundance and are thus inexpensive because it is mostly made from waste products in the shellfish industry (12).

Starch is a polysaccharide that is derived from maize, potato, cassava, and cereal grains. It is made up of linear (amylose) and branching (amylopectin) sections. Because of its numerous advantageous characteristics, such as biodegradability, affordability, abundance, transparency, colorlessness, flavorlessness, tastelessness, diminished water sensitivity, outstanding oxygen barrier properties, renewability, edibility, and its capability to form excellent biofilms, it is considered one of the most promising biodegradable polymers for applications in food packaging. It is frequently regarded as a viable alternative to plastic for food packaging.

In food packaging industry, the use of starch in food wrapping and covering is vindicated due to its non-toxic, odour-free, and neutral nature, along with its excellent film-forming capabilities. Furthermore, starch can serve as a carrier for natural antioxidants and antibacterial agents, enabling the production of intelligent packaging resources. Starch exhibits notable properties such as exceptional barrier and film-making abilities, making it a promising candidate for the production of environmental food packaging resources that can potentially replace petroleum-based synthetic alternatives in the future. Nonetheless, the hydrophilic nature, brittleness, and low mechanical strength of starch-based packaging materials have limited their claim in the food business, particularly in packing and covering (8). To address the issues, various starch alterationmethods are employed to progress functional properties such as package thickness, water content, solubility (S), and swelling index (SI) of starch films (3).

Materials and Methodology

Preparation of plant extract using clove and cinnamon

In this method flower buds of clove and barks of cinnamon were used for the preparation of plant extract. 25 g of cinnamon barks and 25 g of clove flower buds were collected cleaned in running tap water and shade dried. It was then made into coarse powder. 1 liter of distilled water was added to coarsely powdered cinnamon and clove.The mixture was then placed in a shaker at 20°C and agitated at 100 rpm for 24 hours. The resultant extract was then filtered and kept at 4°C.

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Green Synthesis of zinc oxide nanoparticles

25 ml of the plant extract was heated at 60°C for 10 mins. 3g of zinc nitrate hexahydrate was added to the extract and the solution was left for one hour until white precipitate is formed. The resultant solution was then shifted to a crucible, tailed by aeration for 12 hours at 65°C to form a creamy paste. This paste was washed several times with solution of distilled water and ethanol to eliminate impurities. The paste was dried in a furnace at 300°C for 1 hour to synthesize green zinc oxide nanoparticles Table 1.

Development of Zinc oxide nanoparticles incorporated Food Packaging film

Methodology: Zinc oxide nanoparticles embedded starch films were produced and casted on a petri plate to form the food packaging films. For this procedure, 200 ml of deionized water and 6 g of tapioca starch were combined while continuously being stirred at 300 rpm for 15 minutes. 200mg of green synthesized zinc oxide nanoparticles were then added to this solution. 1.8 g of glycerol as plasticizer and 0.5 ml of 1% acetic acid solution (to sustain the pH of the starch mixture) was mixed and heated at 85°C till the solution became gelatinous for 45 minutes. The produced starch content was immediately subjected to a 5-minute sonication at 90°C, followed by vacuum oven degassing. Finally, prepared homogeneous solutions were distributed evenly on the sterilised plates and set aside in an incubator for 24 hours at 25°C for drying purpose. Two types tapioca starch based food packaging films were produced and the description is given in Table 2.

Functional Propertiesof the developed tapioca starch based food packaging films (F1, F2):

The packaging material's functions include physical protection and stability of the film, hence protection of the contained food matter inside. The following properties are tested for both F1 and F2 packaging films.

Moisture content (MC)

Weight loss was used to gauge the films' moisture content (MC). From each film, equal ratio of 2cm wereused, and the results were evaluated. The samples were then dehydrated for 24 hours at 105°C before being evaluated once again.

$$\text{Moisture Content} = (w_a/w_b - 1) \times 100$$

Where w_a is the weight of the films before drying and w_b is weight of the films after drying.

Swelling index (SI)

Swelling index describes how starch and water molecules interact. Briefly stated, samples measuring 2 x 2 were kept for drying

Table 1: Chemicals used for green synthesis of zinc oxide nanoparticles

S. No	Chemical Name	Quantity
1	*Syzygium aromaticum* L (clove)	25 g
2	*Cinnamomum zeylanicum* (cinnamon)	25 g
3	Zinc nitrate- hexahydrate	3 g
4	Ethanol (for washing)	50 ml

Table 2: Synthesized films

Films	Film characteristics
F1	Tapioca starch food packaging film without Zinc Oxide nanoparticles
F2	Tapioca starch food packaging film incorporated with Zinc Oxide nanoparticles

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at 105°C for 24 hours before being measured for weight. Dried samples were submerged in distilled water for two minutes before being taken out. The swollen samples had extra water removed before being weighed.

$$\text{Swelling Index (SI)} = [(A_2 - A_1)/A_1] \times 100$$

Where, A_2, A_1 is the weights of increased samples after the removal of surplusdistilled water and mass of dehydratedfilms respectively.

Solubility (S)

F1and F2 film was split into samples having a 2*2 cm^2 dimension. All prepared samples were weighed (w_0) after drying for 24 hours at 105°C. During storage at 25°C for 24 hours, dehydrated samples was submerged in a container with 15 ml of deionized water. The swollen samples were then taken out, dehydrated at 105°C for another 24 hours weighed (w_1) once more.

$$\text{Solubility (S)} = [(M_1 - M_2)/M_1] \times 100$$

Where, M_1 is the mass of dehydratedfilms before water immersion and M_2 is the dry mass of the unsolvablefilm after absorption.

Characterization of Zinc oxide nanoparticles incorporated tapioca Starch Films

Scanning Electron Microscope analysis (SEM)

The microscopic structure of altered starch and compound films was analyzed via Scanning Electron Microscopy (SEM) with the OMD 2x2 model. The standard procedure was employed for the test, utilizing anfast-tracking voltage of 10 kV. Picturesare captured at intensifications ranging from 300 to 2500 cm^{-1}.

Antimicrobial activity of synthesized zinc oxide nanoparticles incorporated tapioca starch films

a) Antibacterial activity of the film was determined by well diffusion method on Muller Hinton agar medium. The antibacterial activity of Zinc oxide nanoparticles incorporatedtapioca starch film against *E. coli* (gram-negative bacteria) and *S. aureus* (gram-positive bacteria) was investigated using the standard agar diffusion method. The antibacterial test was conducted according to CLSI guidelines (M02-A12) against two bacterial strains.The medium was prepared by dissolving of Muller Hinton agar in deionized water. After cleansing, the media was transferred to petri plates and allowed to solidify for one hour. Once solidified, the inoculum was spread onto the solidified media using a sterile swab moistened with the bacterial suspension. Wells were created using a cork borer. Samples and the positive control, Streptomycin (1 mg/ml - 20 µL), were loaded into the respective wells. The plates were then incubated at 37°C for 24 hours. Microbial growth was assessed by measuring the diameter of the zones of inhibition.

b) The antifungal activity of the sample was assessed using the well diffusion method on Potato Dextrose Agar (PDA) medium. The medium was prepared by dissolving 4.4 g of PDA in 100 ml of distilled water. After sterilization, the medium was poured into sterile petri plates and allowed to solidify for 1 hour. Once solidified, the inoculum was spread onto the plates using a sterile swab moistened with the fungal suspension. Wells were created using a cork borer *candida albicans* and *aspergillus niger* were the two test organisms used. The sample and the positive control, ketoconazole (10 mg/ml - 20 µL), were loaded into the respective wells. The plates were then incubated at 37°C for 24 hours. Antifungal activity was determined by measuring the diameter of the zones of inhibition.

Quantitative analysis of synthesized tapioca Starch based packaging film, wrapped Over Sweet lime segments (F1 and F2 films)

pH

The pH of sweet lime was observed during the storage period using a pH meter during the initial and post storage period of 28 days at 4°C.

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Brix

The pulp was separated from the sweet lime segments and crushed to remove the pulp. The pulp was further squeezed to form filtered fruit juice which was used to measure total soluble solids, which has a series of 0-32%.

Acidity

Acidity was determinedby following the A.O.A.C (2016). Briefly, six grams of sweet lime juice was addedto 25 ml of deionized water in a flask. The juice was titrated with a 0.01 N NaOH solution, with 1% phenolphthalein as an indicator. A pale pink colour in the juice indicated the endpoint of the titration. The acidity was stated as percentage of ascorbic acid per 100 grams of sweet lime pulp.

Vitamin C

10 g of crushed sample was added to 50 ml of the metaphosphoric acid-acetic acid solution to stabilize the ascorbic acid. The mixture is then filtered paper to obtain a clear extract. This solution was then titrated against DCPIP solution until pink colour appears as endpoint. The vitamin C was reported as mg per 100 ml of sweet lime pulp.

Reducing sugars

The fruit juicewasneutralized precisely with concentrated sodium hydroxide (NaOH) and phenolphthalein as an indicator, then diluted to a volume of 100 ml. Thesolution was titrated against the mixture of Fehling's solutions A and B, titrant value was calculated.

Non reducing sugars

10 ml of Fehling's reagent was added to dextrose solution to complete the titration. The solution was boiled for 2 minutes with addition of 1 ml of methylene blue indicator solution. The remaining standard dextrose solution was added until the blue color of the indicator disappears and non reducing sugars value was calculated.

Total sugars

Total sugars is calculated as Total reducing sugar – Non reducing sugar x 0.95 + Reducing sugar (AOAC 2000 16TH edition).

Weight:

The weight of all sweet lime segments in both F1 and F2 packaging was measured initially and finally after 28 days stored at 4°C. This was done to determine the weight loss or weight gain of the packaging materials.

Results and Discussions

Functional properties of the synthesized films (F1 and F2)

The functional properties of tapioca starch based packaging films (F1 and F2) are shown in Table 3.

Moisture content (MC)

The films' ability to contain moisture was expressed by means of moisture content. It also has an impact on the practical qualities including mechanical strength and water vapor penetrability, making it a crucial film quality. *Seligra et al.,* 2014 reported that the MC also improves with an increase in starch concentration. According to *Ghanbarzadeh et al.,2011*, acetic acid passed in between the starch polymer and lowered the contact, which led to an improvement in the MC. The MC is also increased by glycerol or plasticizer concentration, as reported by *Wang et al*. in 2017.

Swelling Index (SI)

According to *M.A. Bertuzzi et al*., 2007, when starch concentration increases,

Table 3: Results of functional properties of the films

Tester films	Moisture content %	Swelling index %	Solubility %
F1	10.21± 0.25	28.37±0.14	21.63 ±0.42
F2	9.98±0.54	27.02±0.35	20.70 ±0.74

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so does the swelling index. According to *Ghanbarzadeh et al., 2011* the contact between thestarch molecules like amylopectin and amylose and the water fragments changes with the quantity of acetic acid and plasticizer in the tapiocastarch packaging film. The green synthesized Zinc Oxidenanoparticles embedded tapioca starch film F2 was found to have lower SI (27.02 ±0.35) than the other starch film F1 without nanoparticles. This might be as a result of the hydrophilic properties that the Zinc Oxidenanoparticles capping on the tapioca starch film has generated.

Solubility

Solubility process provides information on how films interact with water molecules; solubility is crucial in the choice of the right matrix for food. According to research by *Maryam Adilah et al., 2017* the presence of hydrophilic material in the starch films boosted the films' solubility. *Seligra et al., 2016* investigated how the addition of acetic acid reduces the solubility of the tapioca starch film. It has been found that the solubility reduces as Zinc oxide nanoparticles are added in the tapioca starchfilm. Evaluation between the two films i.e., F1 and F2, it was detected that F1 exhibits higher solubility (26.37 ±0.63) than the F2 film. F2 had the minimum solubility i.e., 20.70±0.74(%), because of low hydrophilic nature.

Characterization of the Zinc oxide nanoparticlesnanoparticles embedded starch films

Scanning Electron Microscope Analysis: The microscopic graphs of the starch and nanocomposite films were examined using SEM. (as shown in Fig.1 (a), (b). Fig. 2 (b) at various resolution, showed that the Zinc oxide nanoparticlesincorporated in the starch solution comprising acetic acid (as cross-linker) and glycerol (plasticizer) resulted insmoothscattering of Zinc oxide nanoparticles. SEM images showed Tapioca starch film had small holes on the surface of the film as a result of Zinc oxide nanoparticles adhering to the film's surface. Micrographs also demonstrated that when Zinc oxide nanoparticles were introduced to the starch solution, the film surface formed was found to be uniform and smooth.

Antimicrobial activity of synthesized zinc oxide nanoparticles incorporated tapioca starch films

The starch solution containing ZnO nanoparticles (at a concentration of 20, 40, 60, and 80 mg) was supplemented to wells

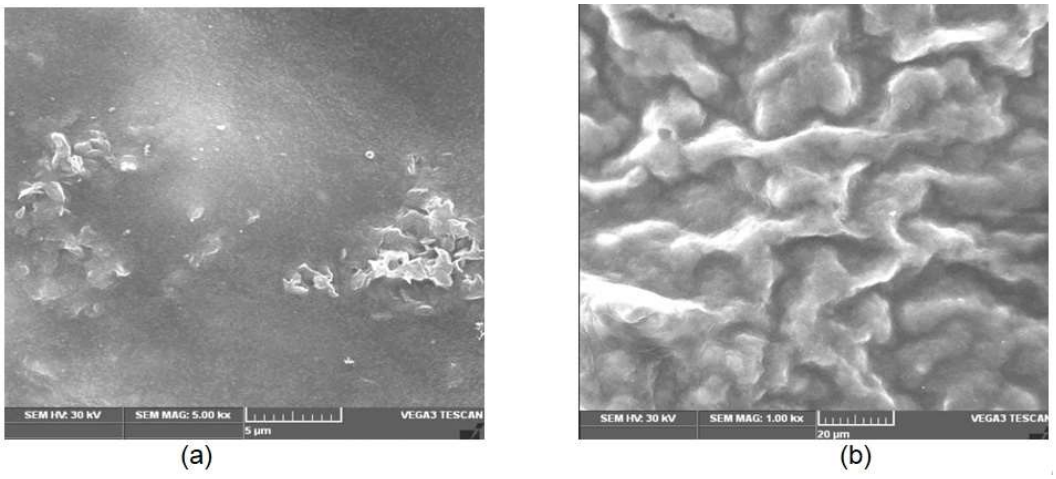

Figure 1: (a) SEM Images of tapioca starch packaging film without Zinc oxide nanoparticles; (b) SEM Analysis of tapioca starch packaging film embedded with Zinc Oxide nanoparticles

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(a) (b)

Figure 2: (a) F1 film wrapped over sweet lime segments, (b) F2 film wrapped over sweet lime segments- initial packaging

Table 4: Antibacterial activity of Zinc oxide nanoparticles incorporatedtapioca starch film					
Microorganisms	Zone of Inhibition (mm)				
	1	2	3	4	5
Staphylococcus aureus	1.2	1.8	4.2	5.7	30
Escherichia coli	7	8.9	10.5	11	22
Note: 1- 20 µl, 2- 40 µl, 3-60 µl, 4- 80 µl 5 -Streptomycin (control)					

Table 5: Antifungal activity of Zinc oxide nanoparticles embedded tapioca starch film					
Microorganisms	Zone of Inhibition (mm)				
	1	2	3	4	5
Aspergillus niger	0.8	0.8	1.2	2.4	30
Candida albicans	0.1	0.3	1.1	1.8	20
Note: 1- 20 µl, 2- 40 µl, 3-60 µl, 4- 80 µl 5 ketoconazole (control)					

(6 mm) on the agar plate. This Zinc oxide nanoparticles embedded starch solution was used as it deliverscomparable results to testing the film directly.

The results, shown in Table 4, indicated that Zinc oxide nanoparticles incorporated tapioca starch packaging films exhibited significant inhibitory activity against *E. coli* (gram-negative bacteria) when compared to *S. aureus* (gram-positive bacteria).

The antifungal activity of the tapioca starch film incorporated with zinc oxide nanoparticles was assessed using the well diffusion method on Potato Dextrose Agar Ketoconazole (1mg/ml -20 microlitre)was used as controlfor comparison.The results, shown in Table 5, indicated that Zinc oxide nanoparticles embedded starch films exhibited higher inhibitory activity against *Aspergillus niger*when compared to *Candida albicans*.

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Quantitative analysis of tapioca starch based packagingfilm (F1 and F2), wrapped over sweet lime segments

The sweet lime segments were separated from the peel individually and then placed over the developed films F1 and F2. These segments were wrapped and stored at 4°C for 28 days. Post 28 days samples were tested for pH, brix, acidity, vitamin C, reducing sugars, non-reducing sugars, total sugars, and weight. weight of test packets were found to be increased both in F1 and F2 packaging after 28 days (Fig. 2A & B, Fig. 3A & B, and Table 6). Theacidity, vitamin-C, total sugars, reducing sugars, non reducing sugars and total sugars contents were recorded to be increased in both F1 and F2 film packaging and was comparatively high in F2 film packaging after 28 days compared to initial fresh fruit segments, An opposite trend was observed for the pH and brix levels of sweet lime segments in different packaging.

(a) (b)

Figure 3: (a) F1 film wrapped over sweet lime segments, (b) F2 film wrapped over sweet lime segments- post 28 days stored at 4°C

Table 6: quantitative parameter of sweet lime segments wrapped over F1 and F2 packaging films

Parameters	Initial (before packing)	28 days in refrigerated storage (4^0C) control (tapioca starch)	28 days in refrigerated storage (4^0C) experimental (tapioca starch with nano ZnO)
pH	4.74	4.80	4.50
Brix	10	10.9	10.3
Acidity %	1.518	1.218	1.483
Vitamin C (mg/100ml)	48.8	24.1	24.9
Reducing sugars %	6.65	6.56	6.62
Non reducing sugars %	6.4	5.5	5.9
Total sugars %	13.21	12.18	12.86
Total weight (sample and packaging) g	Initial	18.8096	19.573
	final	19.1190	20.4501

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Table 7: Antimicrobial activity of sweet lime segments wrapped over F1 and F2 packaging films stored at 4°C for 28 days

Packaging materials	Total bacteria count (CFU/ml)	Total yeast and mould count (CFU/ml)
F1	29×10^{-3} CFU	2×10^{-3} CFU
F2	12×10^{-3} CFU	2×10^{-3} CFU

The brix values were relatively lower, measuring 10.9 in F1 film packaging and 10.3 in F2 film packaging. The slights changes in reducing sugar, (6.56 mg/ml), total sugar (12.18 mg/ml) and non reducing sugar (5.5 mg/ml) of F1 packaging were recorded in comparison to initial fresh segment juice as 6.65, 13.21, and 6.4 mg/ml, respectively. The changes in reducing, non-reducing, and total sugars in the juice were greater in F1 packaging compared to F2 packaging. Both packaging types provided a shelf life of 28 days for the segments under refrigerated storage at 4°C. However, due to overlapping of the segments in the test packages, they became watery and lost their shelf life within 28 days.Total bacterial count on nutrient agar from sweet lime segments juice after 28 day of storage was found to be 290 CFU/ml in F1 packaging filmand 120 CFU/ml in F2 packaging film.Similar findings were reported by *Raccachet al., 2007* on quantitative analysis of Nagpur mandarin segments (Table 7).

Conclusion

The present study involved developing of two starch based food packaging films F1 (tapioca starch based food packaging film), F2(tapioca starch based food packaging film embedded with greensynthesized Zinc oxide nanoparticles) Films functional properties, structural morphology and quantitative assesments were tested. Functional analysis and characterization of synthesized starch films-F1and F2 was performed.Thus, from the whole study, it can be concluded that biofilm developed from tapioca starch (F2) was found to be the better option for food packaging as provided better functional properties and better quantitative and anti microbial results when wrapped over sweet lime segments.

Future Scope of Work

Various biologically synthesised nanoparticles can be studied since green synthesis is more ecological, economical, and useful than chemical synthesis. Moreover, starch films using an improvedblend of cross-linking agents (such as genipin, glutaraldehyde, glyoxal, etc.), plasticizers (such as HPMC, PVA, etc.), and other stabilising agents (such as agar, xanthan gum proteins, emulsifiers, etc.) shall be investigated. The qualitative analysis of films can performed with various forms of highly perishable food products.

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A Cross-Sectional Study on Prevalance of Food Allergy and Its Knowledge Among Malaysian Population

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Abstract

Food allergy can cause severe acute allergic reactions such as hives, rashes and generalized swelling, eczema, diarrhoea, vomiting, stomach aches, asthma and sinusitis. Allergy to food substances may seem common in Malaysia but available data is limited. Since it is detrimental in certain circumstances, hence the awareness regarding allergies is crucial. The objective of this study is to determine the prevalence rate of food allergy, to evaluate the knowledge and awareness of food allergy and to determine the relationship between the status of food allergy and knowledge of food allergy among Malaysia population. The study employed a cross-sectional descriptive study using a validated questionnaire shared with 321 respondents aged 18 years and above from all over Malaysia. Descriptive analysis was conducted to gauge the prevalence while Pearson correlation was used to determine the relationship between status of allergy and knowledge. There were 105 respondents with food allergy and the prevalence rate was 0.42 per 100,000 people which was considerably lower compared to other countries like Korea and US. 19% of respondents answered that food intolerance and food allergy were the same as opposed to a study in US reporting a value of 64.9%. The Pearson correlation analysis showed there were significant relationship between the status of allergy and knowledge level of food allergy ($p<0.05$). The study showed that Malaysian population had a moderate knowledge towards food allergy. This is a cause for concern because lack of knowledge may cause severe consequences when related to food allergy.

Introduction

Food is an essential source of nutrition, comprising of carbohydrates, fats, and proteins. It plays a crucial role in promoting development and growth throughout life. According to the World Health Organization (WHO), a food allergy is defined as an adverse health effect resulting from a specific immune response that consistently occurs upon exposure to a particular food (1). The prevalence of food allergies has increased, particularly among children compared to adults. A food allergy involves an adverse reaction to food through an immunological mechanism (2).

Clinical symptoms of food allergies can range from mild discomfort to severe or life-threatening reactions, necessitating immediate medical intervention. Symptoms may manifest on the body, including redness, itchiness, eczema, hives, rashes, and even asthma. The severity of reactions varies, with some leading to anaphylaxis, a potentially life-threatening response (3).

In Asia, clinicians often perceive the prevalence of food allergies to be low. The types of food allergies differ from those in Western populations, where peanuts and tree nuts related allergies are most common. In Asia, the specific allergens vary by region. For example, bird's nest is a common allergen inducing anaphylaxis in Singapore, while in Southeast Asia, galacto-oligosaccharide-containing formula has been known to trigger allergies in Thailand,

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Vietnam, and Singapore. In India, particularly in regions with a high vegetarian population, legumes such as chickpeas are a common cause of food allergies, and eggplant allergy has a high prevalence (4).

Currently, the treatment for allergies involves medication, lacking preventive measures or a cure. Raising public awareness of food-induced allergies can contribute to a simultaneous decrease in the prevalence of food allergies, ultimately improving the quality of life for affected individuals. Public awareness plays a crucial role in early prevention before allergies arise, especially given the increasing prevalence rates. The relationship between knowledge and the prevalence of food allergies is significant, with better-informed individuals experiencing lower rates. Limited studies exist on knowledge and awareness of food allergies in Asian countries compared to the United States. In Malaysia, awareness, knowledge, and attitudes toward food allergies among food handlers are found to be only moderate (6).

This study aimed to determine the prevalence and knowledge of food allergies among the Malaysian population. Since the prevalence of food allergies in Malaysia is not well-established, this research can provide prevalence rates for adolescents, adults, and the geriatric population. The strong correlation between knowledge and prevalence suggests that increasing public awareness can help reduce the prevalence of food allergies. The study aimed to raise awareness about the importance of understanding food-induced allergies to prevent potential life-threatening syndromes.

Methods

Study Design

This study adopted a cross-sectional descriptive design to evaluate both the knowledge and prevalence of food allergies within the population of Malaysia. The study targeted individuals aged 18 years and older residing in Malaysia. Data collection occurred between April 2020 and May 2020. A pre-validated questionnaire, previously assessed in a pilot study involving 15 respondents conducted in January 2020, was employed for the study.

Ethical Consideration

The study was approved by the Institutional Research Ethical Committee of KPJ Healthcare University, Nilai, Malaysia with the reference no: KPJUC/RMC/SOP/EC/2020/248.

Bias

To mitigate bias, a simple random probability sampling method was employed. The study design aimed to exclusively encompass the target population. The questionnaire, previously validated and checked for reliability in a preliminary study, underwent a thorough review to eliminate any questions that might elicit biased or favourable responses. Additionally, the study achieved a reasonable response rate.

Target Population

The study focused on the public residing in Malaysia as its target respondents. Eligible participants were individuals aged 18 years and above, currently residing in Malaysia, encompassing both citizens and non-citizens. Selection of respondents adhered to specific inclusion and exclusion criteria.

Inclusion Criteria

- Age of 18years old and above
- Competency to understand English and Bahasa Melayu.
- Local resident in Malaysia

Exclusion Criteria

- Age of 17 years old and below.
- Not local resident in Malaysia
- Incomplete questionnaire.

Sampling Procedure

In this study, a convenience sampling method was employed, utilizing a questionnaire format for data collection. The

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questionnaire aimed to assess both the knowledge and prevalence of food allergies within the Malaysian population and was distributed widely across the country. The distribution of questionnaires took place through online platforms, including social media channels such as Facebook and Twitter, as well as through WhatsApp. Prior to participating, respondents were provided with an explanation of the study's objectives, and their voluntary participation involved expressing their personal opinions through marked responses to ensure an understanding of food allergy awareness.

Informed Consent

The online survey form was explained, and informed consent was clearly delivered prior to answering the questionnaire and they are advised to proceed for next section upon consent.

Sample/ Study size

The determination of the sample size for this study was guided by its research objectives, considering the population size. Utilizing the Daniel equation, the calculation yielded an approximate sample size of 280 respondents, which was then chosen for the study. The study aimed to explore the impact of sample size and species characteristics on the performance of various species distribution modelling methods, as highlighted by Hernandez et al. Their findings suggested that larger sample sizes enhance model accuracy.Given the self-administered questionnaire approach for data collection, there was a potential for a high risk of low response rates and errors. To address this concern, and considering the exclusive criteria of incomplete data collection, a 10% additional margin was incorporated into the sample size. Thus, 308 respondents were calculated to compensate for possible withdrawals and incomplete data. In anticipation of a 10% non-response rate, the final adjusted sample size used for the study was 310 respondents.

Study Tools

The quantitative research design employed in this study focuses on objective measurement through the analysis of data collected via a validated questionnaire. The questionnaire was prepared in two languages, English and Bahasa Melayu, adapted from two articles - "A population-based questionnaire survey on the prevalence of peanut, tree nut, and shellfish allergy in 2 Asian populations" (5) and "Australian Parents Food Allergy Knowledge" (6). It underwent preliminary pilot testing on a small sample to validate the questionnaire and gather suggestions for item improvement, a crucial step in determining inclusion or rejection of items for the main study. The pre-validated questionnaire, used as a survey tool for the public, was distributed to 310 respondents, in line with the sample size calculated through Daniel's equation. The self-administered questionnaire was filled out by the respondents themselves. Distribution to the 310 respondents occurred randomly through online platforms, ensuring a self-filled questionnaire where respondents expressed their opinions and provided responses.

Data sources/measurements

The data sources for each variable of interest in this study are the members of the public residing in Malaysia, assessed through a set of questionnaires. The questionnaire comprised of 37 questions, organized into three sections. Section A focused on sociodemographic information and is divided into two parts. Part I included 5 questions related to personal information, while Part II consisted of 4 questions regarding the history of food allergies. In Section B, the questionnaire addressed the prevalence of food allergies among the public, containing 11 questions. Most questions used a Dichotomous scale with the options "Yes" or "No," except for re-challenge questions (5). Section C assessed the knowledge of the public regarding food allergies and included 17 questions with response options such as "Agree," "Disagree," and "Do not know." This section employed a 3-point Likert Scale (6).

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Statistical Analysis

All data analysis for this study was performed using Statistical Package for the Social Sciences (SPSS) software, version 26. Demographic information was analysed using frequency, while the knowledge scores were calculated. Each correct answer in the knowledge section was coded as (3), incorrect answers as (2), and "Do not know" responses as (1). The item scores were then summed to obtain the total knowledge score on food allergies, ranging from a minimum of 17 to a maximum of 48. The total scores were converted into percentages, categorized based on the method used in "Knowledge Attitude and Preventive Behaviors Towards Hand Foot and Mouth Disease Among Caregivers of Children Under Five Years Old in Bangkok, Thailand" (8). Descriptive statistics, including mean, standard deviation, frequency, and percentage, were applied to analyse the demographic information related to food allergies and knowledge. The results were summarized using the Chi-square test. The level of statistical significance was set at a p-value < 0.05, and a confidence interval (CI) of 95% was considered.

Results

The recommended sample size was three hundred and ten participants aged 18 and above. The amount of participants that responded during data collection period was 325. Among the participants, 321 respondents completed the questionnaire.

Sociodemographic data

Personal information

The collected data revealed the age distribution of respondents as follows: 18-28 years old, constituting 233 respondents (72.6%); 29-38 years old, comprising 33 respondents (10.3%); 39-48 years old, with 26 respondents (8.1%); 49-58 years old, accounting for 23 respondents (7.2%); 59-68 years old, with 5 respondents (1.6%); and those above 69 years old, representing the smallest group with 1 respondent (0.3%). The calculated categorical age mean is 2.55. The questionnaire identified two genders, with 79 respondents (24.6%) being male and 242 respondents (75.4%) being female. Regarding race groups from the questionnaire, respondents were categorized into Malay, Chinese, Indian, and Others. The majority were from the Malay population, totalling 227 (70.7%), followed by Indians with 61 (19%), Chinese with 23 (7.2%) respondents, and Others with 10 (3.1%) respondents.

This study encompassed 14 states, focusing on the Malaysian population. Johor had the highest number of respondents at 109 (34%), followed by Perak with 49 (15.3%), Selangor with 37 (11.5%), Kedah with 29 (9%), Pulau Pinang with 24 (7.5%), and Negeri Sembilan with 17 (5.3%) respondents. Four states recorded an equal number of respondents: Perlis and Sabah with 11 (3.4%) each, and Melaka and Sarawak with 6 (1.9%) each. Kelantan, Pahang, Terengganu, and Wilayah Persekutuan Kuala Lumpur collected 7 (2.2%), 8 (2.5%), 4 (1.2%), and 3 (0.9%) respondents respectively.

This study encompassed five education levels: Master's, Degree, Diploma or Foundation, Secondary School, and Primary School. Among the 321 respondents, the highest number held a Degree, accounting for 160 (49.8%) respondents, followed by those with a Diploma or Foundation background, totaling 128 (39.9%). Respondents with a Master's, Secondary School, and Primary School background constituted 5 (1.6%), 27 (8.4%), and 1 (0.3%), respectively. Regarding geographical areas, respondents were classified as urban or rural. The majority, 231 (72%), were from urban areas, while 90 (28%) were from rural areas.

History of food allergy

Regarding food allergy findings, two questions were posed in this study: 1) Whether respondents are aware of food allergies, and 2) If respondents themselves have a food allergy. The majority, 307

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(95.6%) respondents, knew about food allergies, while 14 (4.4%) did not. In terms of having a food allergy, most respondents, 216 (67.3%), reported not having a food allergy, whereas 105 (32.7%) respondents confirmed having one.Concerning family history, 175 (54.4%) respondents had a family history of food allergies, while 146 (45.5%) did not.Among the respondents without food allergies (216 or 67.3%), 60 (18.7%) self-reported having a food allergy, and only 45 (14%) respondents had a doctor-diagnosed food allergy.

Prevalence of food allergies

The respondents were categorized into six groups based on when they first experienced food allergies: between 0 to 10 years old, 11 to 20 years old, 21 to 30 years old, 31 to 40 years old, 41 to 50 years old, and 51 to 60 years old. The highest frequency occurred among respondents who experienced food allergies for the first time between the ages of 11 to 20, totalling 52 (16.9%). Following this, 35 (10.9%) respondents reported their first experience between 0 to 10 years old. Additionally, the first reaction occurred for 8 (2.5%) respondents between 21 to 30 years old, 6 (1.9%) between 31 to 40 years old, 3 (0.9%) between 41 to 50 years old, and 1 (0.3%) between 51 to 60 years old.

Types of Food Allergic

Food allergies can be triggered by various types of food, and respondents in this study were asked to select from a list of potential allergens. The results indicated that the majority of respondents, totalling 67 (20.9%), reported being allergic to shrimp, followed closely by seafood with 65 (20.2%) respondents. Peanut and fish allergies were reported by 15 (4.7%) and 11 (3.4%) respondents respectively. Interestingly, egg allergies and allergies to other types of food (e.g., chocolate, chili) were reported by an equal number of respondents, each with 10 (3.1%). Additionally, meat, fruit, milk, and soy allergies were reported by 9 (2.8%), 5 (1.6%), 4 (1.2%), and 3 (0.9%) respondents respectively. Wheat and yogurt allergies were reported by the fewest respondents, each with 2 (0.6%).

Symptoms of Food Allergic

The analysis of symptoms revealed that the highest number of respondents, 58 (18.1%), experienced redness of the skin. Following this, hives, itchy throat or mouth, running nose or congestion, swollen lip, swollen eye, and abdominal pain were reported by 53 (16.5%), 36 (11.2%), 32 (10.0%), 30 (9.3%), 26 (8.1%), and 14 (4.4%) respondents respectively. Vomiting and other symptoms (e.g., acne, feverish, swollen ear) were reported by an equal number of respondents, each with 11 (3.4%). Wheezing or trouble breathing and diarrhoea were reported by 10 (3.1%) respondents each.

Type of Medication

In the analysis of the type of medication, the majority of respondents, comprising 28 (8.7%), reported taking antihistamines for their food allergies. Some respondents, totalling 12 (3.7%), could not recall the name of the medication. Additionally, anti-inflammatories and other medications (e.g., methotrexate) were reported by 9 (2.8%) and 7 (2.2%) respondents respectively.

Action taken after allergic reaction

During allergic reactions, respondents were presented with three options: 1) seek the doctor, 2) take medication without consulting a doctor, and 3) wait for symptoms to resolve without taking medicine. The majority of respondents, totalling 59 (18.4%), chose to wait for symptoms to resolve without taking medicine. Following this, 49 (15.3%) respondents opted to seek the doctor, and 41 (12.8%) chose to take medication without consulting a doctor.

Ability after allergic reaction

According to the results, the analysis indicated that the majority of respondents, totalling 61 (19.0%), continued to eat the

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food causing their allergic reactions and still experienced symptoms. The remaining respondents either did not eat the allergenic food or consumed it without experiencing symptoms, with total numbers of 25 (8.7%) and 16 (5.0%) respectively.

Knowledge towards food allergy

According to the findings, the level of knowledge was categorized into three levels: poor knowledge (<60%), moderate level (60 to 80%), and good knowledge (>80%). The majority of respondents, totalling 158 (49.2%), scored at a moderate level. This was followed by those with good knowledge, comprising 139 (43.3%) respondents, and those with poor knowledge, accounting for 24 (7.5%) respondents.The frequency and percentage of answers given by the respondent based on the provided statements are available in Table 1.

The association between the status of allergy and the knowledge level

The results indicated an association between respondents having a food allergy and their level of knowledge on food allergies. Among the respondents without food allergies, the majority, totalling 105 (32.7%), demonstrated higher knowledge of

Table 1: The frequency and percentage of answer given by the respondent

Statement	Respondents, n (%)
Food allergy involves the immune system (T)	216 (67.3)
Food allergy is a serious health problem in Malaysia (T)	115 (35.8)
Are the peanut allergy being the most common allergies in Malaysia? (T)	169 (52.6)
The only way to know that you are allergic to a food is with a medical test. (F)	114 (35.5)
Are the rashes being the most common symptoms of food allergy? (T)	232 (72.3)
People with food allergies are treated differently because of their food allergy. (T)	202 (62.9)
Eczema may be the first sign of having a food allergy. (T)	143 (44.5)
Asthma is an important risk factor for severe anaphylaxis (T)	195 (60.7)
Food additives are common food allergens? (eg: colouring agent, preservative) (F)	60 (18.7)
Milk allergies is similar to milk intolerance. (F)	61 (19.0)
Teenagers are at higher risk for fatal food allergy compared to younger children. (T)	57 (17.8)
Taking a daily allergy medicine can prevent food allergy reactions. (F)	114 (35.5)
Are medicine could cause an allergic reaction? (T)	249 (77.6)
Do we need to check all the ingredients contained in the food before buying it? (T)	287 (89.4)
For someone who has a food allergy, staying away from the food that allergic to him/her is difficult. (T)	203 (63.2)
People with food allergies worry a lot about their allergy. (T)	240 (74.8)
It is difficult for people with food allergies to safely eat at restaurant. (T)	227 (70.7)
N = frequency	

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food allergies, in contrast to 11 (3.4%) respondents with food allergies having a poor knowledge level. The Chi-Square value was 8.165 with a p-value of 0.017 ($p<0.05$), suggesting animportant relationship between respondents having a food allergy and their level of knowledge on food allergies. Consequently, it can be concluded that knowledge is influenced by the presence or absence of food allergies among respondents.

Discussion

The study focused on the prevalence of food allergies among Malaysians aged 18 and above, as well as their knowledge and awareness regarding this issue. The research aimed to determine the correlation between the prevalence of food allergies and the level of knowledge among the population.

Prevalence of Food Allergy

Based on the findings, the prevalence of food allergies among the Malaysian population was determined to be 0.42 per 100,000 people. In comparison with other Asian countries, previous studies mainly focused on the pediatric population, where the prevalence among children aged 5 to 12 years ranged from 4 to 5%, and in Korea, it was found to be 10% among those aged 6 to 12 years (9). A study reported an incidence of food allergies in Spain at 4.6% and in Australia at 19.1% (10). Similarly, In the United States, the prevalence of food allergies is approximately 3.7% among adults and 6% for infants and young children (11). Therefore, when compared to these studies, the prevalence of food allergies in Malaysia appears to be considerably lower.

This study identified twelve types of food allergens, with shrimp being the most common cause of allergies at 20.9%, followed by seafood at 20.2%. This was followed by peanut (4.7%), fish (3.4%), egg (3.1%), meat (2.8%), fruit (1.6%), milk (1.2%), soy (0.9%), wheat (0.6%), and yogurt (0.6%). In comparison, a study in the United States in 2017 reported shellfish allergies at 3.9%, with peanut (2.4%), tree nut (1.9%), and fin fish (1.1%) following as the highest reported allergens (12). Additionally, a 2019 report indicated that shellfish allergies were highest among adults at 2.9%, affecting an estimated 7.2 million adults in the US, followed by milk, peanut, tree nut, fin fish, egg, wheat, soy, and sesame (13). These results align with previous studies that have identified fish and seafood as common causes of allergies, particularly shellfish like lobster, shrimp, crab, and crayfish. However, some discrepancies exist, as Vierk et al. identified milk or dairy as the most common allergen, attributing it to misconceptions between milk intolerance and milk allergy (14). This study suggests that milk was a moderate cause of food allergies, differing from previous findings that included individuals with lactose intolerance.

This study explored various patterns of food allergens across different countries, particularly in Asia. It revealed that shellfish, including prawn or shrimp, exhibited high prevalence rates in Asian countries such as the Philippines (5.12%) and Singapore (5.23%), contrasting with the lower prevalence in the United States (0.7%) (4). Despite peanut being a significant allergen, its prevalence was comparatively lower in Asian countries compared to Western countries. The study conducted in Malaysia, an Asian country, highlighted fish and seafood as major causes of allergies, potentially influenced by geographic and genetic factors given the diverse ethnicities in the Asian population (15).

The study's results indicated that a majority of respondents experienced redness of the skin and hives during allergic reactions, with the severity of symptoms influenced by factors like the amount of food ingested, age, and absorption speed. In Vietnam, hives were the most frequent symptom, followed by diarrhea (16). Contrarily, studies in other regions identified allergic rhinitis as the most common symptom, followed by urticaria and asthma (17, 18). Skin manifestations were common

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among adults, varying in severity based on the allergen and ingested amount.

Regarding medication during allergic reactions, the most commonly used were antihistamines and anti-inflammatories, including loratadine, cetirizine, chlorpheniramine, hydrocortisone, prednisolone, and betamethasone. These medications align with standard practices for managing and relieving food allergy symptoms, emphasizing the importance of complete allergen avoidance and pharmacotherapy, including rapid-acting oral antihistamines (levocetirizine, cetirizine, loratadine, fexofenadine, and mizolastine) and, when necessary, Epipens containing adrenaline for IgE-mediated reactions (19; 20). Oral corticosteroids are employed in managing eosinophilic esophagitis and gastroenteritis symptoms, while topical corticosteroids, though less effective, are used in specific cases (21). The treatment received by respondents aligned with established protocols for addressing allergic reactions and alleviating symptoms.

Knowledge of food allergy

Another objective of this study was to assess the knowledge of food allergies among the Malaysian population. The findings indicated that the majority of respondents had a moderate level of knowledge regarding food allergies. This contrasts with a study in the United States, where adult respondents showed poor knowledge regarding the distinction between food allergy and food intolerance, with only 64.9% answering correctly (21). Gupta et al. (22) also found wide variations in knowledge among the general public, with many misconceptions related to the prevalence, definition, and triggers of food allergies.In this study, only 61 out of 321 respondents incorrectly identified food intolerance and food allergy as the same, similar to the misconception found in Gupta et al.'s study (21). Therefore, the level of knowledge about food allergies among the Malaysian population appears to be somewhat similar, especially when examining each knowledge item individually.

When comparing parents with food allergies, Gupta et al. found that knowledge was 75% correct, but parents were unaware that teenagers are at a greater risk of fatal anaphylaxis than younger children (23). In contrast, this study revealed that only 17.8% of respondents agreed with this statement, indicating a lack of awareness about the increased risk of anaphylaxis in teenagers. However, when focusing on each knowledge item, the majority of respondents correctly acknowledged that food allergic reactions involve the immune system (67.3%). This aligns with the pathology of food allergies, where immune reactions are triggered by food protein antigens (24). Moreover, in line with Zukiewicz-Sobczak et al.'s findings that allergic reactions can be caused by medications, ranging from mild to severe skin or systemic reactions, 77.6% of respondents in this study correctly agreed that medicines can cause allergies (25).

Relationship between the status of allergy and level of knowledge of food allergy

The analysis of the association between allergy status and the level of knowledge of food allergies in this study revealed a noteworthy difference between non-allergic and allergic respondents. The majority of non-allergic respondents demonstrated good knowledge. This finding contrasts with a previous study by Allan (7), which found no major difference in parents' knowledge of food allergies based on their child's allergy status. Similarly, a study by Choi, Ju, and Chang found no major difference in knowledge levels based on students' allergy status in the age range of 6 to 12 years (26).In contrast, the present study focused on the allergy status of the general public, revealing a difference between those with allergies and those without in terms of knowledge of food allergies. The higher number of non-allergic respondents in this study may have influenced the association between allergy status and knowledge level. This discrepancy suggests that the context and scope of the study

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population can impact the relationship between allergy status and knowledge of food allergies.

Conclusion

The present findings assessed the knowledge and awareness of food allergies in the Malaysian population, revealing a moderate level of knowledge. Recognizing the difference between allergies and intolerance, understanding the treatment of food allergies, and identifying allergy symptoms are crucial aspects of public awareness. Notably, this study found that the status of food allergy considerably impacted knowledge scores. Non-allergic respondents demonstrated higher knowledge levels compared to those with allergies, emphasizing the importance of understanding these distinctions within the population.

Limitations

Establishing causality is challenging in this study due to its cross-sectional design. The approach involved distributing questionnaires online to the public, but faced challenges in reaching a representative sample across all 14 states in Malaysia. Understanding the queries posed a difficulty for some respondents, leading to potential loss in the number of participants. Additionally, a few respondents did not take the questionnaire seriously, posing a threat to the reliability and validity of the data analysis. Efforts were made to overcome these challenges by personally explainingthe study's purpose to respondents. Despite these challenges, the study was conducted in accordance with the necessary requirements.

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Conflict of Interest

We declare that we have no conflict of interest.

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Multivariate Calibration Techniques Using UV Spectrophotometry for Quantifying Ticagrelor in Pharmaceutical Formulations

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Abstract

The aim of this project is to create and verify a straight forward, highly responsive, and precise UV spectrophotometric technique for measuring the amount of ticagrelor in both its raw form and its pharmaceutical form. This will be achieved by utilizing multivariate linear regression analysis. Multivariate linear regression analysis was performed to assess the correlation between concentration and absorbance. Absorbance values were collected at five different wavelengths, and the resulting data were utilized to develop a predictive model for quantifying ticagrelor in pharmaceutical formulations. This analysis facilitated the identification of key wavelengths that contribute to accurate concentration measurements were analyzed using statistical methods. The implemented method exhibited linearity across a concentration range of 5-15 µg/mL, with a correlation coefficient valuation of 0.998. The peak absorption wavelength (λmax) of Ticagrelor was detected at 257 nm. The percentage RSD values for intraday and interday precision were found to be within the ICH recommendations' acceptable range of 2%, namely in the ranges of 0.540 - 0.558 and 0.540 - 0.565, respectively. The created approach was determined to be straightforward, expeditious, precise, and reliable in accordance with the International Council for Harmonisation (ICH) criteria Q2 (R1). Utilizing statistical methods ensures accurate and consistent results, unaffected by instrument errors or experimental variables.Since the drug's absorbance is measured at five different selected wavelengths, the multivariate calibration methodology has been said to be more reliable than the other published procedures. This led to the development of a quick and easy method based on mathematical building blocks.

Keywords: Ticagrelor, Multivariate Linear Regression Analysis, Validation, UV Spectrophotometry and ICH

Introduction

Ticagrelor is a notable compound characterized by its chemical name, (1S,2S,3R,5S)-3-[7-[[(1R,2S)-2-(3,4-difluorophenyl)cyclopropyl]amino]-5-propylsulfanyltriazolo[4,5-d]pyrimidin-3-yl]-5-(2-hydroxyethoxy)cyclopentane-1,2-diol[1]. It has the molecular formula C23H28F2N6O4S and a molecular weight of 522.568 (Figure 1). This drug belongs to the antiplatelet category, specifically acting as an oral antagonist of the adenosine diphosphate (ADP) receptor P2Y12. It inhibits this receptor in a reversible and direct manner, providing a faster. Ticagrelor works by preventing the aggregation of platelets, a process crucial for the formation of blood clots. By doing so, it reduces the risk of heart attacks and strokes[2]. It achieves this by inhibiting the P2Y12 receptor on platelets, which plays a key role in their activation and aggregation.Ticagrelor is widely used in the treatment of patients with coronary artery disease who are at risk of heart attacks or strokes[3]. It is particularly effective in individuals who have experienced a myocardial infarction or have acute coronary syndrome. Studies have shown that

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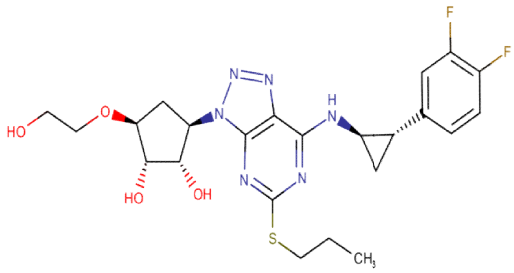

Figure 1: Chemical Structure of Ticagrelor

ticagrelor is effective in preventing first-time heart attacks or strokes in high-risk patients. Several analytical methods have been established for the quantification of ticagrelor in pharmaceutical formulations and biological samples[4]. These include:

1. Liquid Chromatography-Mass Spectrometry (LC-MS)
2. High-Performance Liquid Chromatography (HPLC)
3. Thin Layer Chromatography (TLC)
4. Fourier Transform Infrared Spectroscopy (FTIR)
5. Ultra-Performance Liquid Chromatography (UPLC)
6. High-Performance Thin-Layer Chromatography (HPTLC)
7. Reverse Phase High-Performance Liquid Chromatography (RP-HPLC)
8. Ultraviolet Spectroscopy (UV)

These methods are essential for ensuring the quality, efficacy, and safety of ticagrelor in clinical use. They allow for precise measurement and monitoring of the drug in various formulations and biological fluids.

Most labs employ spectrophotometric methods as their preferred method because to their low cost, accuracy, precision, and reproducibility. The preferred approach is predicated on a high level of precision and accuracy on the direct estimate of Ticagrelor[5].The method is easy to use and reasonably priced, and it may be applied to examine Ticagrelor. The proposed approach outlines the evaluation of Ticagrelor in pharmaceutical formulations through a UV spectral multilinear regression methodology, utilizing fundamental mathematical principles. Multilinear regression, which extends the concept of a single dependent variable to incorporate multiple dependent variables into the calibration model, enhances the model's flexibility and applicability. This statistical technique is particularly valuable under optimal experimental conditions, as it provides substantial sensitivity and resolving power at a relatively low cost, making it suitable for routine quality control analysis[6].

To ensure the reliability and accuracy of the developed method, it is recommended to validate the approach according to the guidelines set forth by the International Conference on Harmonization (ICH). Specifically, the ICH Q2 (R1) guidelines for analytical method validation should be followed to confirm the method's validity and robustness[7].

The current work aimed to establish a speedy, easy-to-use, accurate, precise, sensitive, and fast analytical method for measuring some of the previously described sophisticated analytical techniques of Ticagrelor[8]. A based on the aforementioned claim, a straightforward analytical method based on UV spectrophotometry enabled multivariate calibration process was recommended to be developed[9].

Materials and Methods

Chemicals and solvents employed

- 0.1M NaOH.
- BRILINTA TABLETS – (Label claim – 90 mg, 10 mg and 20 mg of Ticagrelor), manufactured by Samarth Life Sciences Pvt. Ltd., The marketed tablet formulations were procured from the local market.

Solubility

- Insoluble in ethyl acetate.
- Sparingly soluble in methanol .
- Freely soluble in water, 0.1M NaoH, 0.1M HCl.

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Instrumentation

- UV-Vis double beam Spectrophotometer (Lab India UV- 3092).
- Electronic balance (SHIMADZU AY-220H).

Method development

Selection of solvent

Ticagrelor was reported to be freely soluble in 0.1M NaoH, which was employed as the solvent to solubilize the drug for the entire analysis.

Preparation of standard stock solution

After carefully weighing 10 mg of ticagrelor, it was added to a 100 mL volumetric flask. 50mL of the solvent was added in order to dissolve the contents of the volumetric flask. The solvent was thoroughly combined (1 mg/mL) with the final volume, which was 90 mL. Pipetting 3 mL of the aforementioned solution into a 30 mL volumetric flask, the solvent was used to build up the remaining volume to the appropriate level and the mixture was thoroughly stirred. The resultant solution was further diluted with the solvent to achieve concentrations between 5 and 15 µg/mL.

Determination of λ_{max}

The solvent was employed to dilute the standard stock solution of Ticagrelor to a concentration of 10µg/mL. The UV range of 400-200 nm was used to scan this solution. The UV spectra of Ticagrelor is shown in Figure 2 and the absorbance maxima of Ticagrelor was found to be 257 nm. Five wavelengths were selected in and around the absorbance maxima of Ticagrelor, such as 253, 255, 257, 259 and 261 nm for the study.

Preparation of standard solution for linearity

For linearity testing, the solvent was used to further dilute the Ticagrelor standard stock solution to create concentrations of 5, 7.5, 10, 12.5, and 15 µg/mL.

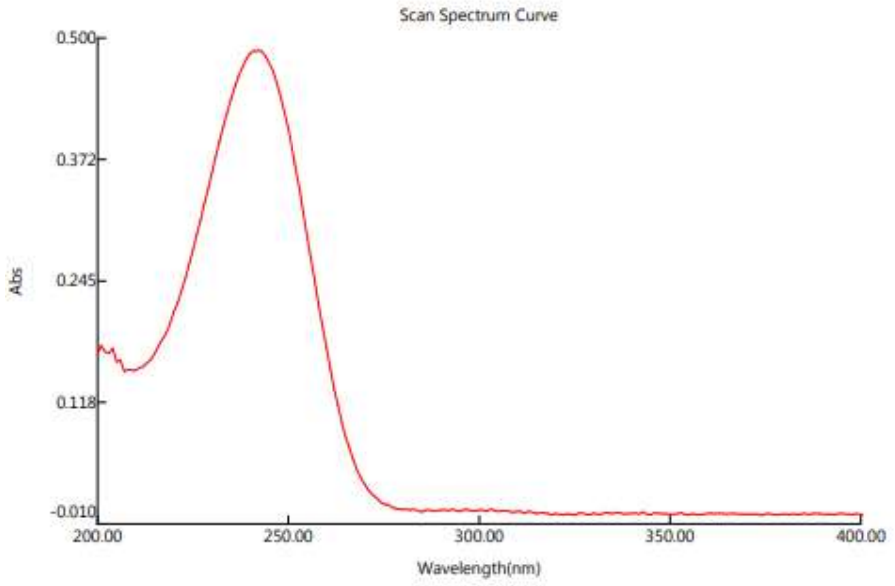

Figure 2: UV spectra of Ticagrelor

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Preparation of sample solution

20 tablets of Ticagrelor (BRILINTA TABLETS – Label claim – 90 mg of Ticagrelor) were measured for weight, and the average weights were established. The tablets were ground into a fine powder and thoroughly combined with the contents. After weighing out 10 mg of Ticagrelor from the combined substance and dissolving it in 90 mL of solvent for 25 minutes using sonication, the solvent was used to bring the volume up to 90 mL. The aforementioned mixture was thoroughly blended and filtered. For additional analysis, the filtrate was appropriately diluted.

Method validation

The developed approach was validated in accordance with the ICH Q2 (R1) protocol, which examined validation parameters such as linearity, precision, and accuracy.

Linearity

To establish a linear correlation and minimize instrumental fluctuations, absorbance measurements were taken for the prepared linearity concentrations at five different wavelengths surrounding the drug's maximum absorbance at 257 nm. These wavelengths were 253, 255, 257, 259, and 261 nm, as detailed in Table 1. The overlay UV spectra, illustrating the linearity at these wavelengths, are presented in Figure 3. Separate correlation coefficient values for the linear regression equations at each of these five wavelengths were calculated and are provided in Table 2. Additionally, Figure 4(a & b) displays the multivariate calibration linearity obtained at these five wavelengths, as well as the cumulative absorbance.

Precision

To evaluate intraday and interday precision, the absorbance of a linearity solution at a 100% concentration (10 µg/mL) was measured across all five wavelengths. For intraday precision, the designated concentration was scanned six times within a single day, while for interday precision, it was scanned across three different days. Figures 5 and 6 illustrate the overlay UV spectra for the intraday and interday precision studies, respectively. The absorbance values obtained at the specified wavelengths for these precision experiments are detailed in

Table 1: Absorbance values at five selected wavelengths

Concentration (µg/mL)	Absorbance				
	253 nm	255 nm	257 nm	259 nm	261 nm
5	0.278	0.284	0.282	0.281	0.277
7.5	0.415	0.423	0.427	0.427	0.415
10	0.543	0.553	0.563	0.557	0.549
12.5	0.692	0.69	0.712	0.711	0.696
15	0.825	0.836	0.848	0.849	0.825

Table 2: Linearity data showing statistical parameters at all five wavelengths

Wavelength	Slope	Intercept	Regression equation	r^2
253	0.0548	0.0026331	y= 0.0548x + 0.0022	0.9996
255	0.0567	0.0032249	Y=0.0567x - 0.0004	0.9998
257	0.0551	0.0023875	y= 0.0551x + 0.0016	0.9997
259	0.0548	0.0031411	y= 0.0548x + 0.0088	0.9996
261	0.0568	0.0026833	y= 0.0568x - 0.003	0.9998

Multivariate Calibration Techniques

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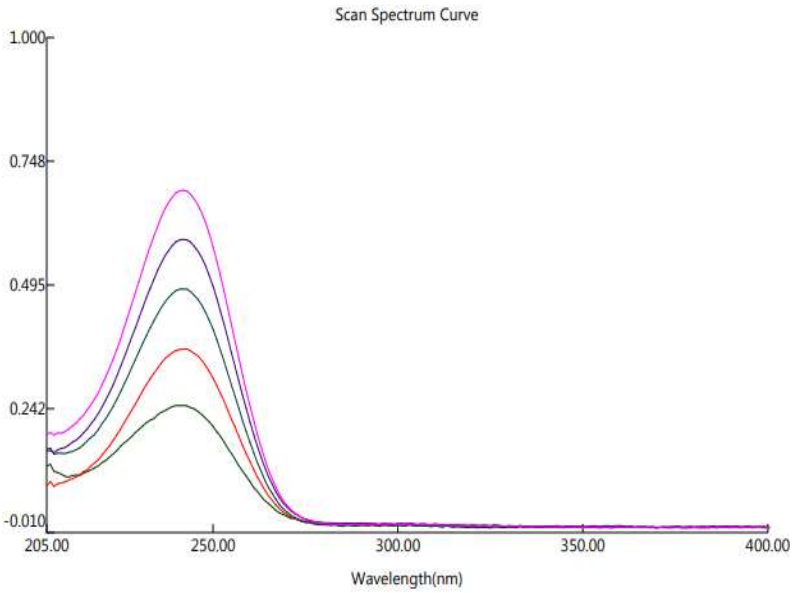

Figure 3: Separate correlation coefficient values

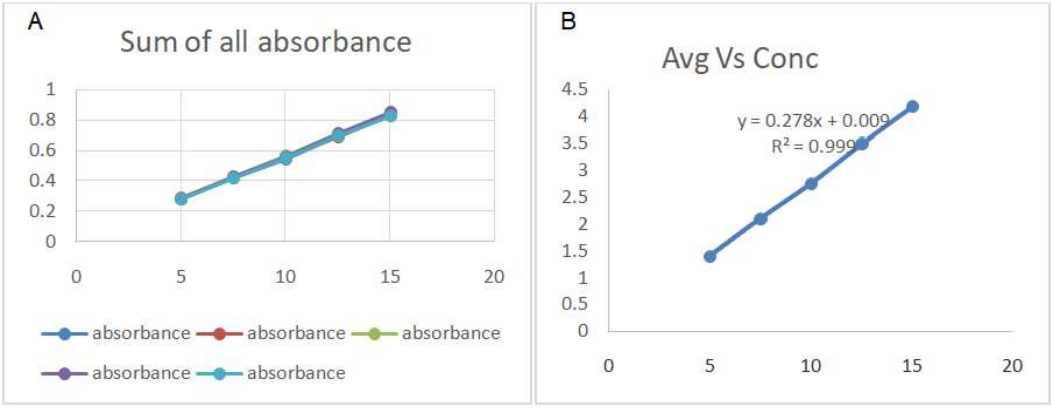

Figure 4: Multivariate Calibration linearity graph (a) and Sum of all Absorbance; and (b) Average *vs* Concentration of Ticagrelor

Tables 3 and 5. Additionally, the calculated standard deviation (SD) and percentage relative standard deviation (% RSD) values for both intraday and interday precision are provided in Tables 4 and 6, respectively.

Accuracy

The accuracy of the developed methodology was evaluated at concentration levels of 50%, 100%, and 15% by recovery studies conducted using the standard addition method. Three distinct 10 mL volumetric flasks were filled with 0.1 mL of the sample solution, and 0.1, 0.5, and 0.7 mL of the standard stock solution were pipetted into the aforementioned volumetric flasks, respectively, from the prepared stock solutions of standard and sample. The final

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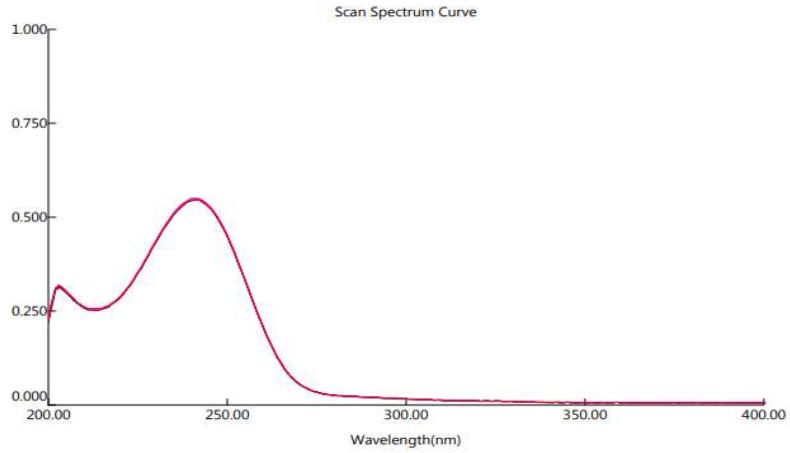

Figure 5: Overlay UV Spectra of Ticagrelor showing intraday precision studies

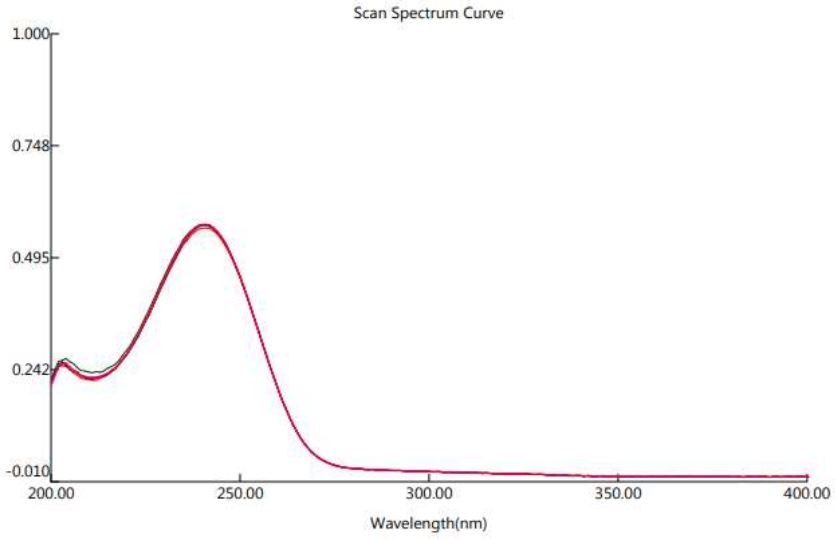

Figure 6: Overlay UV Spectra of Ticagrelor showinginterday precision studies

volume was raised to the necessary level using methanol. It was decided what the recovery percentages were. Table 7 presents a summary of the recovery investigations' results, while Figure 7 shows the overlay UV spectra that demonstrate correctness.

Assay

The absorbance of the extracted sample solutions were recorded at 257 nm. The amount of drug present in the formulations was calculated and the assay results are tabulated in Table 8.

Multivariate Calibration Techniques

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Table 3: Intraday precision at five selected wavelengths

Concentration (µg/mL)	No. of Repetitions	Absorbance (nm)				
		253nm	255nm	257nm	259nm	261nm
10	1	0.544	0.564	0.548	0.548	0.556
	2	0.543	0.563	0.549	0.553	0.557
	3	0.546	0.562	0.547	0.548	0.556
	4	0.543	0.563	0.547	0.548	0.553
	5	0.546	0.564	0.546	0.556	0.555
	6	0.546	0.565	0.548	0.548	0.553

Table 4: Intraday Precision of TICAGRELOR showing Mean, SD, and % RSD

Conc(µg/mL)	Description	253 nm	255 nm	257 nm	259 nm	261 nm
10	Mean	0.544	0.563	0.547	0.548	0.554
	SD	0.001378	0.001049	0.000816	0.00216	0.001722
	% RSD	0.253151	0.186124	0.149086	0.393727	0.310436

Table 5: Interday precision at five selected wavelengths

Conc (µg/mL)	No. of Repetitions	Absorbance (nm)				
		253 nm	255 nm	257 nm	259 nm	261 nm
10	1	0.543	0.563	0.549	0.553	0.557
	2	0.544	0.564	0.548	0.548	0.556
	3	0.543	0.563	0.547	0.548	0.553
	4	0.546	0.562	0.547	0.548	0.556
	5	0.545	0.564	0.547	0.547	0.554
	6	0.546	0.565	0.548	0.548	0.553

Table 6: Interday Precision of Ticagrelor showing Mean, SD, and % RSD

Wavelength (nm)	Amount present (µg/mL)	Amount added (µg/mL)	Amount recovered (µg/mL)	% Recovery
253	6	4	9.8	98.00
		9	10.2	102.00
		14	19.6	98.00
255	6	4	9.9	99.00
		9	14.8	98.67
		14	19.8	99.00
257	6	4	9.8	98.00
		9	15.2	95.00
		14	19.6	98.00

(*Contd.*)

Table 6: Interday Precision of Ticagrelor showing Mean, SD, and % RSD

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Wavelength (nm)	Amount present (µg/mL)	Amount added (µg/mL)	Amount recovered (µg/mL)	% Recovery
259	6	4	9.9	99.00
		9	15.5	96.88
		14	19.6	98.00
261	6	4	9.8	98.00
		9	14.89	98.90
		14	21	100.8

Table 7: Recovery studies of Ticagrelor at five selected wavelength

Concentration (µg/mL)	Description	253 nm	255 nm	257 nm	259 nm	261 nm
10	Mean	0.544667	0.5635	0.5475	0.550167	0.555
	SD	0.001506	0.001049	0.001049	0.003488	0.001673
	% RSD	0.276416	0.186124	0.191563	0.634003	0.301499

Table 8: Assay of Ticagrelor in marketed pharmaceutical formulations

Label claim (mg)	Amount estimated (mg)	%Assay	Average (n = 3)	SD	% RSD
TICAGRELOR (90MG)	89.98	99.98	99.99	0.0231	0.231
	90.01	100.01			
	89.97	99.97			

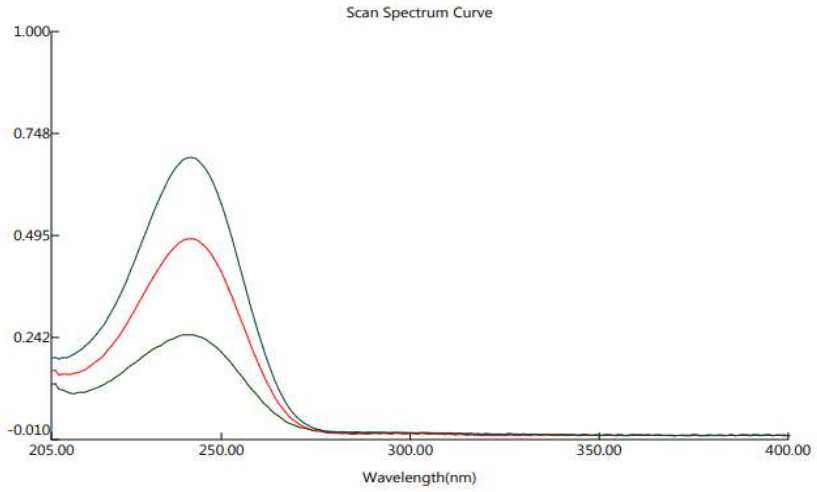

Figure 7: Overlay UV Spectra of Ticagrelor showing Accuracy

Multivariate Calibration Techniques

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Results and Discussion

The absorption maxima of Ticagrelor were observed at 257 nm using 0.1M NaOH as solvent.

Linearity

Within the defined concentration range of 5– 15 µg/mL, the devised technique was found to be linear. All five wavelengths of 253, 255, 257, 259 & 261 were used to construct a linear regression equation. The resulting correlation coefficient values were determined to be more than 0.998.

Precision

Both intraday and interday precision investigations were conducted. The percentage RSD values for intraday and interday precision were found to be within the ICH recommendations' acceptable range of 2%, namely in the ranges of 0.540 - 0.558 and 0.540 - 0.565, respectively. The low estimated percentage RSD number demonstrates the established approach's accuracy. The mean, standard deviation, and percentage RSD (relative standard deviation) are shown for five different wavelengths in Tables 5 and 6.

Conclusion

For evaluating ticagrelor in pharmaceutical formulation, it was discovered that the established quick and easy UV spectrophotometric-assisted Multivariate calibration approach was linear, sensitive, accurate, and precise. Since the drug's absorbance is measured at five different selected wavelengths, the multivariate calibration methodology has been said to be more reliable than the other published procedures. This led to the development of a quick and easy method based on mathematical building blocks. It is highly recommended for routine quality control testing of Ticagrelor in pharmaceutical formulations as it is more predictable than previous spectrophotometric procedures.

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Computer System Validation of UV Spectroscopy Instrument Software (CARRY UV) using V Model

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Abstract

Computer System Validation (CSV) is a critical aspect of ensuring that any software or system used in regulated environments, such as pharmaceuticals, healthcare, and biotechnology, meets its intended purpose and operates consistently within specified parameters. The UV spectroscopy instrument, specifically the CARRY UV software, is a pivotal tool in analytical laboratories for measuring the absorbance and transmission of ultraviolet and visible light by a sample. Validating this software using the V-Model ensures its reliability, accuracy, and compliance with regulatory standards. The V-Model, or Validation Model, is a systematic approach widely used in software development and validation. It emphasizes verification and validation activities corresponding to each stage of the software development lifecycle. Key aspects include accuracy in measurement, data integrity, user access controls, and audit trails. This stage involves outlining the software's capabilities, such as wavelength range, data processing algorithms, user interface design, and integration with laboratory information management systems (LIMS). The design specifications phase involves creating a blueprint for the software's architecture. This includes the design of databases, software modules, and user interfaces. For CARRY UV software, it is crucial to ensure that the design supports robust data handling, secure user access, and accurate data processing. For the CARRY UV software, developers must focus on implementing algorithms for accurate spectral data analysis and ensuring the software's compatibility with various hardware configurations. Integration testing involves combining individual modules and testing them as a group. System testing validates the complete and integrated software to ensure it meets the specified requirements. For the CARRY UV software, this involves testing the entire workflow from sample measurement to data analysis and reporting. It ensures the software performs reliably under different conditions and usage scenarios Users test the CARRY UV software in a real-world environment to ensure it performs as expected. This phase includes installation qualification (IQ), operational qualification (OQ), and performance qualification (PQ) to ensure the CARRY UV software is installed correctly, operates according to specifications, and performs consistently in the production environment. Throughout the validation process, meticulous documentation is maintained. This includes validation plans, test scripts, test results, and validation reports. Documentation is essential for demonstrating compliance with regulatory requirements, such as those set by the FDA, EMA, or other relevant authorities. Validating the CARRY UV software using the V Model ensures a structured and thorough approach to verifying and validating the software's functionality, performance, and compliance. This methodical process helps identify and mitigate risks early, ensuring the software's reliability and integrity in critical analytical applications. Through rigorous testing and documentation, the V Model supports the delivery of a robust and compliant UV spectroscopy instrument software, ultimately enhancing laboratory efficiency and accuracy.

Keywords: Installation Qualification, Operational Qualification, & Performance Qualification, quality, safety, identity, efficacy

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Introduction

Computer system validation is a meticulous and well-documented process that ensurescomputer-based systems will generate information and data that meet predetermined requirements [1-3]. This validation process is crucial in pharmaceutical companies and medical device industries as it helps to enhance the handling of complexities and system performance. The primary objective of computer system validation is to guarantee accuracy, consistency, reliability, and consistent performance of the system in line with predefined specifications [4-7]. In the pharmaceutical industry, computer system validation plays a pivotal role in improving product quality, streamlining processes, and supporting the production of high-quality products. One of the major advantages of validating computer systems is the support it provides for quality controls, ensuring that processes are followed correctly and reducing the likelihood of manual errors [8-9]. To maintain industry standards, both the European Medicines Agency (EMA) and the Food and Drug Administration (FDA) have issued guidelines for Computer System Validation (CSV) practices.Computer system validation is a unique and essential process that maximizes effectiveness and enhances the overall quality in the pharmaceutical Indus.

Methods and Materials

Instruments required

1. UVSpectroscopy
2. Software(carryUV)
3. Computer

Installation qualification (IQ)

The purpose of the Installation Qualification is to verify and document that all the key aspects of the hardware and software installation, including operating system details adhere to approved Design Specifications manufacturer's recommendations and environmental conditions. An IQ protocol shall be prepared and will define the level of validation required. The IQ protocol may be separate for hardware and software in the (Tables 1 & 2).

Operational qualification (OQ)

The purpose of the OQ is to verify and document that the individual and integrated components of the System perform reliably and consistently within specified operating ranges as stated in the functional specification. OQ testing will be based on the Impact Assessment. OQ testing shall be conducted in a production environment or a validation environment that has been demonstrated to be equivalent tothe production environment. An OQ protocol shall be prepared for each of the Systems and will define the level of verification required (Table 3).

Performance qualification (PQ)

The purpose of the PQ is to challenge the fully configured release of the System in its normal integrated environment. A protocolshall be prepared which will verify the performance of the System in accordance with the approved URS, Standard Operating Procedures and relateddocumentation.

Testing will be developed to challenge the System as it is used and operated under routine conditions and environmental parameters. This includes the review of each procedure that interfaces with the System and provides evidence that the procedures are in existence, current, applicable and being followed. Sections of the PQ can be incorporated into the OQ (Tables 4 & 5).

Functional risk assessment:

Functional risk assessments should be used to identify and manage risks to patient safety, product quality, and data integrity that arise from failure of the function under consideration.

Functions which impact on patient safety, product quality, and data integrity are identified by referring to the URS, functional specification document (FSD), and the output of the initial risk assessment. Risk

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Table 1: Software Categories

Category	Description	Validation Approach	Typical Example
Category-1 Infrastructure Software	Layered Software used to manage the operating environment	Record version number, verify correct installation by following approved installation procedures.	• Operating Systems • Database Engines • Middleware • Programming Languages • Statistical Packages • Spreadsheets • Network Monitoring Tools • Scheduling Tools
Category-2 Non Configured Software	Run Time Parameters may be entered and stored, but the software cannot be configured to suit the business process	Abbreviated life cycle approach: URS, Risk based approach to supplier assessment, Record version number, verify correct installation, Risk-based tests against requirements as directed by use. Procedures in place for maintaining compliance and fitness for intended use.	• Firmware based applications • COTS software • Laboratory Software • PLC
Category-3 Configured Software	Software, often very complex, that can be configured by the user to meet the specific needs of the user's business process. Software code is not altered	Life Cycle Approach: Risk- based approach to supplier assessment, Demonstrate supplier has adequate QMS, Some life cycle documentation retained only by supplier (e.g. Design Specification). Record Version Number Verify	• LIMS • Data Acquisition System • SCADA • ERP • DCS • BMS • HMI
		Correct installation. Risk based testing to demonstrate application works as designed in the test environment. Risk-based testing to demonstrate application works as designed within the business process. Procedures in place for maintaining compliance and fitness for intended use. Procedures in place for managing data.	

(*Contd.*)

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Table 1: Software Categories

Category	Description	Validation Approach	Typical Example
Category-4 Custom software	Software Custom designed and coded to suit the business process	Same as configurable, Plus: More rigorous supplier assessment, with possible supplier audit. Full Life cycle (FS, DS, Structural Testing, etc.) Designand Source Code Review.	• Internally and Externally developed IT Applications. • Internally and externally developed process control Applications. • CustomLadderLogic. • Spreadsheets-Macro.

Table 2: Installation qualification document

Availability of hardware software configuration					
Step No.	Name document	Expected result	Actual result	Results Pass/Fail	Verified By Name (project trainee)
1.	Instrument name	UV visible spectrophotom eter	UV visible spectrophotomet er	Pass	Vignesh
2.	Make	Agilent Technologies	Agilent technologies	Pass	Vignesh
3.	Model	Carry 3500 UV vis	Carry 3500 UV vis	Pass	Vignesh
4.	Serial number	MYD00473	MYD00473	Pass	Vignesh
5.	System ID	APRD/AD/0009	APRD/AD/0009	Pass	Vignesh
Verification of client software					
1.	Log into system	System allow user to log in	System allow user to log in	Pass	Vignesh
2.	Windows activation	Activation available	Activation available	Pass	Vignesh
3.	Date and time synchronization	Date and time Synchronized with calibrated Master clock	Date and time Synchronized with calibrated Master clock	Pass	Vignesh
4.	Click the Windows Startbuttonthen (All) Programs, Agilent and Carry UV	Windows Start button then (All) Programs, Agilent and Carry UV Available	Windows Start button then (All) Programs, Agilent and Carry UV Available	Pass	Vignesh

(*Contd.*)

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Table 2: Installation qualification document

Verification of client software					
Step No.	Name document	Expected result	Actual result	Results Pass/Fail	Verified By Name (project trainee)
5.	The first time the Carry UV software is open a Software Registration dialog willappear.Click Next	Carry UV software Registration dialog will appear. Click Next is appear	Carry UV software Registration dialog will appear. Click Next is appear	Pass	Vignesh
6.	Complete all the fields on the 'Customer Details' page. Click Next.	Its appear 'Customer Details' page. Click Next.	Its appear 'Customer Details' page. Click Next.	Pass	Vignesh
7.	Complete all the fields on the 'Work Environment Details' page. Click Register.	Its appear 'Work Environment Details' page. Click Register	Its appear 'Work Environment Details' page. Click Register	Pass	Vignesh

Table 3: Operational qualification document

Verification of audit trail					
Step No.	Procedure	Expected result	Actual result	Results Pass/Fail	Verified By Name (Project trainee)
1.	Log in the System administrator ID and Password	System should allow to Login the administrator	System should allow to Login the administrator	Pass	Vignesh
2.	Obtain the audit log of all the transactions executed by the user in this protocol along with Login and Logout history capturing the below information (but not limited to) • UserID /Name • Date/time of run • Original value	Audit trail content should match transaction and activity performed on the system.	Audit trail content should match transaction and activity performed on the system.	Pass	Vignesh

(*Contd.*)

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Table 3: Operational qualification document

Verification of audit trail					
Step No.	Procedure	Expected result	Actual result	Results Pass/Fail	Verified By Name (Project trainee)
3.	Verify that audit trail cannot be turned off/ there is no option for the user to turn off the audit trail.	User should not be able to turn off the audit trail.	User should not be able to turn off the audit trail.	Pass	Vignesh
4.	Try to edit the audit trail	Application should not allow to delete audit trail	Application should not allow to delete audit	Pass	Vignesh
5.	Try to delete the audit trail	Application should not allow to delete audit trail	Application should not allow to delete audit	Pass	Vignesh

Table 4: Verification of Backup

Step No.	Procedure	Expected result	Actual result	Results Pass/Fail	VerifiedBy name (Project trainee)
1.	Login the system Administrator ID and Password	System should allow to Login Administrator	System should allow to Login Administrator	Pass	Vignesh
2.	Verify the data backup	Data backup should take place	Data backup should take place	Pass	Vignesh
3.	Verify that user can be able to access & take data backup	Administrator only should have the access and authorization to take data backup	Administrator only should have the access and authorization to take data backup.	Pass	Vignesh

Assessment consists of identification of risks and the analysis and evaluation of risks associated with system.Risk Identification- is a systematic use of information to identify hazards referring to the risk question or problem description. Risk Analysis- Risk analysis is the estimation of the risk associated withthe identified hazards. It is the qualitative or quantitative process of linking the likelihood of occurrence and severity of harms (Tables 6-8).

Results and Discussion

In order to UV spectroscopy instrument software (CARRYUV) using V Model URS, FRA, IQ, OQ & PQ Tests were

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Table 5: Performance qualification document	
Severity–Rating	
Value	Severity(S)
1	Legligible: • Temporary and in significant impact on GxP requirements which can be mitigated without change to Computer system and within existing procedures.
2	Marginal: • Minor failure, not noticeably affecting functional quality of the computerized system, however, are likely to result in a minor deviation from GxP requirements.This can be mitigated with verification.
3	Catastrophic. • Direct and significant impact on data security / integrity / GxP requirements. A failure that could reasonably result in a safety issue (potential harmto worker) shall be considered as Catastrophic.Designand implementation review to be done with corrective and preventive actions. Risk assessment shall be performed. Review of testing protocols and revision (if necessary) to intensify testing in the failed components. Review and possible revision of impacted SOPs.
4	Critical: • Acritical failure that mayrender the system in operable or result in significant reduction in performance of the computerized system and/or quality of the product or having an impact on data security/integrity GxP requirements. • These risks shall be investigated with corrective and preventive actions, review of testing protocols and revision (if necessary) to intensify testing in the failed components. Review and possible revision of impacted SOPs. Risk assessment shall be performed.
5	Moderate: • Moderate failure likely resulting in reduction in performance of computerized system or quality of the product. These failures are likely to result in a major deviation from GxP requirements. • Risk assessment shall be performed and investigation with corrective action plan shall be derived to mitigate the risks (if any).

done according to the protocol. During the execution of protocol, the tests of the UV Spectroscopy Instrument Software (CARRY UV) using V Model URS, FRA, IQ, OQ & PQ were mentioned the process as follows:

The key process parameters like FRA, IQ, OQ & PQ tests passes all steps and final results are with acceptance limit of user requirement specification. Under user requirement specification all steps are done and all are meet their specifications.

Table 6: Probability–Rating	
Value	Probability (p)
1	Rare, Failure is unlikely
2	Unlikely, Relatively few failures
3	Possible, Occasional failures
4	Likely, Repeated Failures
5	Almost certain, Failure is almost in evitable

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Table 7: Detectability– Rating	
Value	Detectability(D)
1	Very high, will almost certainly be detected
2	High, has a good chance of detecting the risk
3	Moderate, a potential risk maybe detected
4	Low, the risk is unlikely to be detected
5	Very low, the risk will not be detected

Functional risk assessment (FRA). The risks is calculated according to this risk assessment calculation and finds risk is in minor level and it iscorrected and recovered. All tests under Installation qualification (IQ) is passed all tests Including Hardware and Software verification tests are matching with URS. All tests under operational qualification (OQ) is passed all tests Including system security and verification backup are matching with URS. All tests under Performance qualification (PQ) is passed all tests including Performance qualification procedures and performance qualification test plan are matching with URS. Handling of discripancies and risk assessment mitigation action tests

Table 8: Qualitative or quantitative process of linking					
Severity (S) Probability (P)	Negligible (1)	Marginal (2)	Moderate (3)	Critical (4)	Catastrophic (5)
Almost certain(5)	5	10	15	20	25
Likely(4)	4	8	12	16	20
Possible(3)	3	6	9	12	15
Unlikely(2)	2	4	6	8	10
Rare(1)	1	2	3	4	5

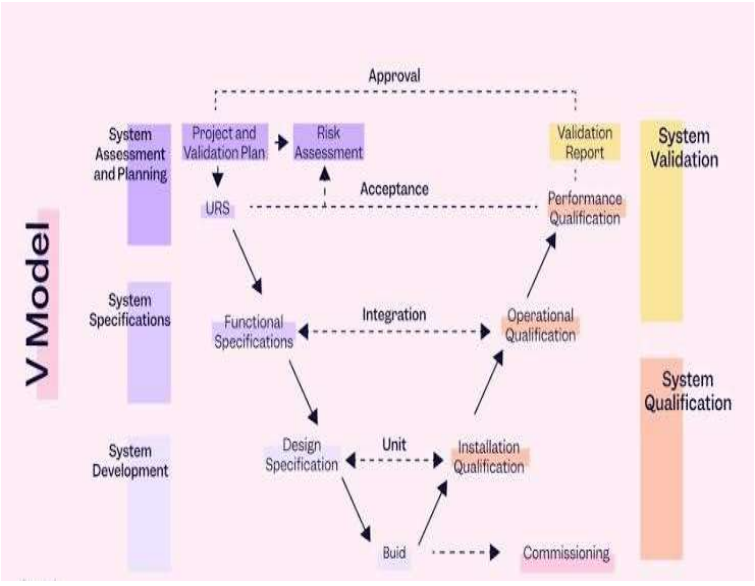

Fig 1: V Model

Computer System Validation

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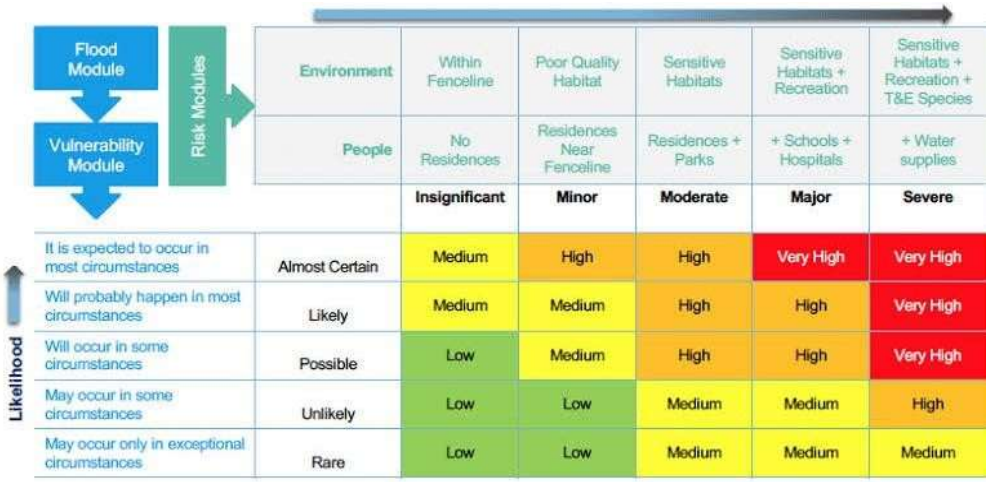

Fig 2: Key Process Parameters

are found with in the acceptance limit. So all, tests are passed and meeting their URS (Figures 1 and 2).

Conclusion

The system must be validated according the Quality System and approved protocols to provide user were data integrity, security and traceability. The computer system validation of UV spectroscopy instrument software will be assessed by using V model. Based on the summary UV spectroscopy instrument software (CARRY UV) using V model were completed successfully. UV spectroscopy instrument software (CARRY UV) using V model were performed and meets their URS and provide quality products as output.

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