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Analysis of Leaf Extract of *Zingiber officinale* by a Hybrid Analytical Technique

Ambily PG[1], Jane Mathew[1]*,Sudhina M[2]

AMbily PG, Lisie College of Pharmacy,Vennala High School Road,Kochi,Ernakulam-682028,Kerala
[1*]NGSM Institute of Pharmaceutical sciences, Nitte (Deemed to be University), Paneer, Deralakatte-575018,Karnataka State
[2]Yenepoya college of pharmacy & research centre, University Road, Deralakatte Mangalore, Karnataka -575018

*Corresponding author: janej@nitte.edu.in

Abstract

There are number of phytoconstituents present in plants, that have the potential to prevent and cure certain human illness. *Zingiber officinalis*, a perineal herb used for culinary purposes and alternative medicine is said to possess antioxidant, antidiabetic, anti-inflammatory, anticancer, antirheumatic, antibacterial and antifungal properties. Though much work has been carried out on the rhizome, there has been limited work done on the leaves of the ginger plant. The qualitative phytochemical analysis of methanolic and aqueous extract of ginger leaves shows the presence of flavonoids, saponins, phenolic compounds and tannins in methanolic and aqueous extract. GC-MS analysis further validates the presence of 13 phytochemical constituents such as caryophyllene, phytol, lanosterol, pentadecanoic acid methyl ester and other secondary metabolites in the methanolic extract, and 13 phytochemical constituents in aqueous extract. The leaf extract was screened for Invitro anti-inflammatory activity by inhibition of protein denaturation method and membrane stabilization test. The % inhibition at 300µg/ml of aqueous extract was observed at 64.88%, 66.09% and 50.57% in Bovine Serum Albumin denaturation method, egg albumin denaturation and Haemolytic RBC membrane stabilization method respectively. In case of methanolic extract maximum percentage inhibition was observed at 300µg/ml was 61.30%, 63.09% and 46.74% in BSA denaturation method, egg albumin denaturation and HRBC membrane stabilization method respectively.

Keywords *Zingiber officinale,* GC-MS, In vitro anti inflammatory

Introduction

Ginger is a herbal spice and folk medicine . Zingiber officinale is a perineal plant with swollen underground stem or rhizomes belonging to the family Zingiberacae(1). The Leaves are lanceolate, glaberous and sessile having a prominent midrib. Its rhizomes are thick lobed, fleshy, ovate, laterally compressed bearing short oblique branches. It is pale yellow to buff in colour with longitudinal striations. Flowers are tiny yellowish green coloured, solitary, in oblong cylindrical spikes. The calyx consists of fused sepals which have three teeth at the apex with the corolla tube being cylindrical, three lobed, greenish, sub equal and lanceolate(2). The fruits are oblong capsules containing oval seeds. Depending on the amount of cork removed ginger can be either ‘scraped’ or ‘unscraped(3). Rhizomes are fiberous in nature. It is having an agreeable odour and pungent taste hence it is used as a spice(4).

Ginger is believed to have origin from India and South-East Asia. Ginger and its components

are known to have various beneficial medicinal properties due the presence of gingerol and 6-shogaol and other active constituents (5,6). It is widely used in a variety of foods because of its nutritional composition and flavouring compounds. Major constituents present are carbohydrates, lipids, terpenes and phenolic compounds. Ginger rhizomes are rich source of vitamins, minerals and iron(7,8). Pre-clinical studies prove that the plant is used for diarrhoea(9), diabetes(10), obesity, allergies, pain(11) and various forms of cancer(12,13). Plant extracts contain number of bioactive molecules which have medicinal properties and are used in formulating ayurvedic preparations(14-16). The ginger rhizome contains non volatile pungent constituents like gingerol and shagaol which possess potent anti inflammatoryproperties(17).

Gas Chromatography - Mass Spectrum (GC-MS) is an analytical technique useful for determination and identification of biologically active constituents present in plant extract (18-21).

The intention of study was to known the phytoconstituents in the methanolic leaf extract of zingiber officinale by preliminary phytochemical screening, and to identify each individual compound by GC-MS,Since there was no literature available whether the leaves of the ginger plant has anti-inflammatory activity. Invitroanti inflammatory studies were carried on the methanolic leaf extract of the ginger plant.

Materials and Methods:

GC-MS analysis of the methanol extract of leaf was performed using Perkin Elmer,the GC model was Clarus 680, and mass spectrometer was clarus600 (EI),and software TurboMass ver5.4.2.

The Clarus 680 GC was used in the analysis employed a fused silica column, packed with Elite-5MS (5% biphenyl 95% dimethylpolysiloxane, 30 m × 0.25 mm ID × 250µm df) and the components were separated using Helium as carrier gas at a constant flow of 1 ml/min. The injector temperature was set at 260°C during the chromatographic run. The 1µL of extract sample injected into the instrument the oven temperature was as follows: 60 °C (2 min); followed by 300 °C at the rate of 10 °C min−1; and 300 °C, where it was held for 6 min. The mass detector conditions were: transfer line temperature 230 °C; ion source temperature 230 °C; and ionization mode electron impact at 70 eV, a scan time 0.2 sec and scan interval of 0.1 sec. The spectrums of the components were compared with the database of spectrum of known components stored in the GC-MS NIST (2008) library.

Collection of plant material

The leaves of Zingiber officinale were collected in and around Thrissur Kerala, in the month of June - August 2020. The collected plant was authenticated by Dr.Raju Krishna Chalannavar Professor and Chairman, Department of Applied Botany, Mangalore University, Mangalagangothri - 574 199.

Preparation of extract

The collected leaves were cleaned; shade dried and coarsely powdered using electrical blender. The extraction was carried out by cold maceration method using methanol and water as solvents. 200gm of powder was macerated with 2.3 L of methanol for 7 days with occasional stirring and extract is filtered through muslin cloth. The filtrate was concentrated under controlled temperature (45^0C -50^0C) and pressure (55 PSIg) using Rotavap rotary evaporator (model: PBU-6D). The total yield obtained after maceration was 25 g of extract.

For the preparation of aqueous extract, 100g of powder was macerated with 1.2L distilled water for 24 hrs. Extract is filtered through muslin cloth and concentrated by evaporation on water bath at temperature of 60^0C – 70^0C and yield obtained was 8g. Both crude extracts were stored in desiccator for further studies.

Preliminary phytochemical analysis

The phytochemical analysis of the extracts was performed as per the standard procedures described by Trease and Evans(22). The extract was evaluated for alkaloids, flavonoids, triterpenoids, steroids, glycosides, carbohydrates, saponins, phenolic compounds and tannins and results are mentioned in Table 1.

Screening of anti-inflammatory activity:

Inhibition of protein denaturation method:

Bovine serum albumin method (*23):* Various concentration of test extract was prepared from the stock solution 100mg/100ml (1000µg/ml). 0.5ml – 3ml of solutions were pipette out and makeup to 10ml in 10ml standard flask using solvents (methanolic extract- methanol and aqueous extract- distilled water).

The reaction mixture was prepared using 0.5ml test extract (50-300µg/ml) and 1% of aqueous solution of bovine albumin. 0.1N HCl was employed to maintain the pH of the above solution. Sample mixture will contain 0.45 ml bovine serum albumin and 0.05 ml of the extract solution.0.05ml of the sample mixture was taken in the test tubes and incubated at 37°C for 20min and then to the above sample mixture 2.5 ml of saline phosphate buffer was added. The absorbance of the sample mixture is taken at 660nm. Same procedure was repeated for standard drug – Diclofenac, by preparing different concentration (50 - 300µg/ml) of the drug. The experiment was performed in triplicates.

Egg albumin denaturation method(24):

Sample solution was prepared using 0.45 ml of 5% egg albumin solution and 0.05ml of the test extract (50-300µg/ml) solution. The sample solutions were taken in test tubes and these tubes were incubated for about 20 min at 37°C. 2.5 ml of the phosphate buffer saline was added to the above solution after incubation period. Further the reaction mixture was heated for 5min at 70^0C. Mixture was cooled and absorbance was taken at 660nm . Diclofenac was used as standard drug, procedure was repeated thrice.

Membrane stabilization test by Heat induced haemolyticmethod(25):

Suspension of red blood cells: 8ml of blood samples were collected from healthy human volunteer who have not been administered with NSAID agents for 14 days before the start of the experiment. Alsever's solution used to prevent coagulation of blood is mixed with gently with equal quantity of blood sample. Transferred to centrifuge tubes and centrifuged for 10 min at 3000 rpm, repeatedly washed thrice with equal quantity of normal saline. The volume of blood was measured and restored as 10%v/v suspension with normal saline.

Heat-induced haemolytic method: Into the centrifuge tube 1ml of test sample of different concentration (50-300µg/ml), and 1 ml of test 10% RBC suspension was taken as reaction mixture. In the control test tube, sample is absent only saline is used. The tubes were incubated at 56^0C for 30min in a water bath. The cooled reaction mixture in the tubes were centrifuged at 2500 rpm and the absorbance of supernatants was recorded at 560nm. Aspirin was the standard drug. The experiment will be repeated thrice for all the test extract samples. The percentage membrane stabilization activity was determined.

Results and Discussion:

Preliminary phytochemical studies were carried out on the methanolic extract of ginger leaves,and showed the presence of flavonoids, triterpenoids, glycosides, saponins, phenolic compounds and tannins are reported in Table 1. Based on these phytochemical constituents,GC-MS analysis was carried on both aqueous and methanolic extracts. The phytochemical constituents present were confirmed from the retention indices, molecular formula, molecular weight (MW) and peak area in percentage in comparison with the data available from the GC-MS NIST (2008) library.

The GC-MS data of methanolic and aqueous extract are displayed in tables 2 & 3 and figures1& 2 respectively. The anti inflammatory effect of leaf extract of zingiber officinale by bovine serum albumin denaturation, egg albumin denaturation and HRBC membrane stabilization method are reported in tables 4,5 & 6 respectively and the graphical representation is shown in figures 3,4 & 5 respectively.

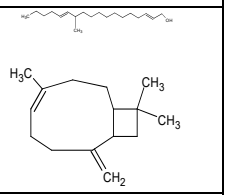
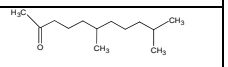
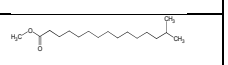
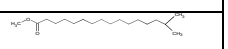
Table1: Preliminary phytochemical test of the methanolic leaf extract of *Zingiber Officinale*

Sl.NO	Test for	Results
	Alkaloids	
	Dragendroff's	-ve
	Hager's	-ve
	Wager's	-ve
	Mayer's test	-ve
	Reducing sugars	
	Molish's	-ve
	Benedict's	-ve
	Fehling's	-ve
	Tollen's	-ve
	Flavonoids	
	Shinoda	+ve
	Alkaline test	+ve
	Terpenoids	
	Libermann-Burchard's test	+ve
	Glycosides	+ve
	Resins	-ve
	Saponins	+ve
	Steroids	
	a) Libermann-Burchard's test	+ve
	b) Salkowaski test	+ve
	Tannins	+ve
	Phenolic compounds	+ve

Discussion

Studies have proved that certain phytoconstituents present in plants, such as, the presence of volatile oils, flavonoids, xanthones and triterpenoids have similar mechanism of action. For the development of better therapeutic agents such constituents, need to be isolated and tested, so as to reduce the pathologies related in-

Table 2: GC-MS results of phytochemical constituents in *Zingiber officinale* leaves methanolic extract

Sln o	Retenti on time	Peak area %	Name of compound	Molecular formula	Molecul ar weight	Structure
1	14.208	4.243	12-Methyl E,E-2,13-octdecadi-1-ol Caryophyllene	$C_{19}H_{36}O$ $C_{15}H_{24}$	280.5 204	
2	17.869	1.263	2-undecanone 6,10-dimethyl	$C_{13}H_{26}O$	198	
3	18.165	6.714	1-Octadecyne	$C_{18}H_{34}$	250	
4	18.650	20.527	Pentadecanoic Acid-14-methyl-, methyl ester	$C_{17}H_{34}O_2$	270.450 7	
5	19.560	1.181	Hexadecanoic acid 15 methyl-methyl ester	$C_{18}H_{34}O_2$	284	

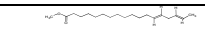
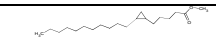
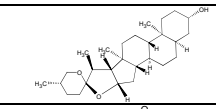
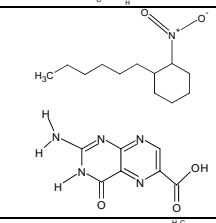
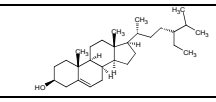
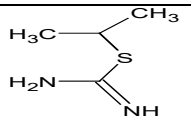
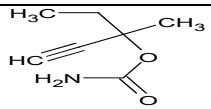
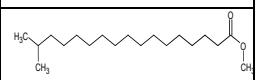
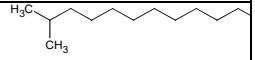
6	19.825	39.206	17-Octadecynoic acid, methyl ester 1-Hexadecyne	$C_{19}H_{34}O_2$ $C_{16}H_{30}$	294.47 222 .902	
7	21.631	1.500	Eicosanoic acid, methyl ester	$C_{21}H_{42}O_2$	326.557 0	
8	22.381	7.612	13,16-octa decadicynoic acid, methyl ester	$C_{19}H_{34}O_2$	294.47	
9	22.941	2.462	Cyclopropanepentanoic acid 2-undecyl	$C_{20}H_{38}O_2$	311	
10	26.588	7.128	Sarsapogenin	$C_{27}H_{44}O_3$	416.6	
11	27.623	3.590	1Hexyl- 2- nitrocyclo hexane Pterin-6-carboxylic acid	$C_{12}H_{23}O_2N$ $C_7H_5N_5O_3$	213 207	
12	28.814	2.857	Beta sitosterol	$C_{29}H_{50}O$	414.762 4	
13	29.404	1.712	Heptacosanoic acid, methyl ester	$C_{28}H_{56}O_2$	424	

Table 3: GC-MS analysis of aqueous extract of *Zingiber officinale* leaves

Sl	**Retention time**	**Peak area %**	**Compound name**	**Molecular formula**	**Molecular weight**	**Structure**
1	6.880	11.812	Carbamimidothioic acid, 1-methylethyl ester	$C_4H_{10}N_2S$	118.198	
2	7.585	0.884	1-pentyn-3-ol,3-methyl-carbamate	$C_7H_{11}NO_2$	141	
3	18.575	10.683	Oxirane (hexadecycloxymethyl) Heptadecanoic acid,16 methyl,methyl ester	$C_{19}H_{38}O_2$	298 298	
4	19.065	33.265	15-Methyl hexadecanoic acid		270	

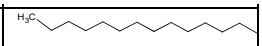
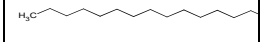
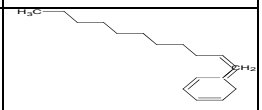
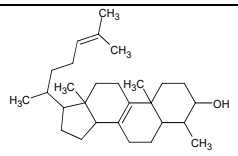
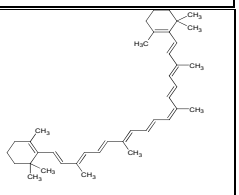
5	20.350	5.74	1-hexadecyne	$C_{16}H_{30}$	222	
6	20.471	28.839	1-octadecyne	$C_{18}H_{34}$	250	
7	27.173	1.147	Z,Z,Z,1,4,6,9 non adecatetraene	$C_{19}H_{32}$	260	
8	27.253	0.829	4-pentyl bicyclohexyl-4-carbonamide		279	
9	27.408	0.775	Cholesta-8,24-diene-3-ol 4-methyl-(3-beta,4-alpha)	$C_{28}H_{46}O$	398	
10	27.523	1.724	4-pentyl bicyclohexyl-4-carbonamide Pterin-6-carboxylic acid		279 207	
11	28.689	0.985	4-pentyl bicyclohexyl-4-carbonamide Pterin-6-carboxylic acid		279 207	
12	28.714	1.131	4-pentyl bicyclohexyl-4-carbonamide Pterin-6-carboxylic acid		279 207	
13	30.680		Beta carotene	$C_{40}H_{56}$	536	

Table 4: Effect of aqueous and methanolic *Zingiber officinale* leaves extracts on bovine serum albumin denaturation

Tested material	Concentration (µg/ml)	% Inhibition on BSA denaturation ±SEM	IC_{50} value
Diclofenac	50 100 150 200 250 300	35.11±0.59 41.66±0.59 51.19±0.59 58.33±0.59 66.66±0.59 72.61±0.59	178.78
Aqueous extracts of *Zingiber officinale*	50 100 150 200 250 300	29.16±0.59 35.11±0.59 45.83±0.59 52.38±0.59 59.52±0.59 64.88±0.59	202.75
Methanolic extracts of *Zingiber officinale*	50 100 150 200 250 300	27.38±0.59 31.54±0.59 42.26±0.59 50.00±1.03 55.95±0.59 61.30±0.59	213.38

All values are expressed in terms of ± SEM and are found to be significant when compared to control P<0.05

Table 5: Effect of methanolic and aqueous *Zingiber officinale* leaves extracts on egg albumin denaturation

Tested material	Concentration (µg/ml)	% Inhibition on BSA denaturation ±SEM	IC_{50} value
Diclofenac	50 100 150 200 250 300	31.54±0.59 41.66±0.59 49.40±0.59 65.47±0.59 70.83±0.59 74.40±0.59	172.33
Aqueous extracts of *Zingiber officinale*	50 100 150 200 250 300	22.61±1.57 39.88±0.59 48.80±0.59 55.95±0.59 61.90±0.59 66.09±1.03	193.35
Methanolic extracts of *Zingiber officinale*	50 100 150 200 250 300	27.38±0.59 38.69±0.59 47.02±0.59 51.78±1.03 58.33±0.59 63.09±0.59	202.86

All values are expressed in terms of ± SEM and are found to be significant when compared to control P<0.05

Table 6: HRBC membrane stabilization test effect on Zingiber officinale leaves

Tested material	Concentration (µg/ml)	% Inhibition on BSA denaturation ±SEM	IC_{50} value
Diclofenac	50	15.45 ± 0.024	151.93
	100	26.08 ± 0.041	
	150	37.92 ± 0.024	
	200	50.72 ± 0.041	
	250	62.56 ± 0.024	
	300	70.53 ± 0.024	
Aqueous extracts of *Zingiber officinale*	50	18.43 ± 0.186	276.24
	100	24.11 ± 0.741	
	150	30.21 ± 0.852	
	200	36.80 ± 0.155	
	250	44.44 ± 0.268	
	300	50.57 ± 0.352	
Methanolic extracts of *Zingiber officinale*	50	15.67 ± 0.142	295.60
	100	22.11 ± 0.741	
	150	27.47 ± 0.537	
	200	35.91 ± 0.350	
	250	41.37 ± 0.358	
	300	46.74 ± 0.208	

All values are expressed in terms of ± SEM and are found to be significant when compared to control P<0.05

Fig 1: GC-MS chromatogram of methanolic extract of *Zingiber officinale* leaves.

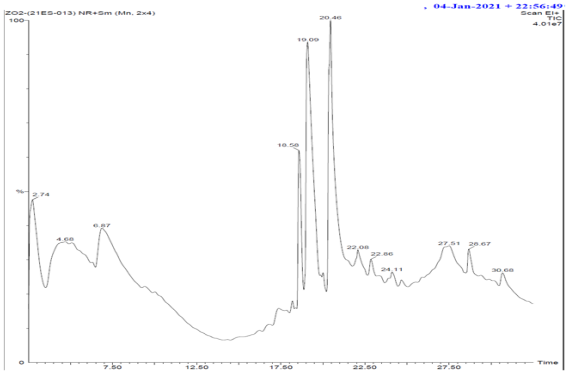


Fig 2: GC-MS chromatogram of aqueous extract of *Zingiber officinale* leaves

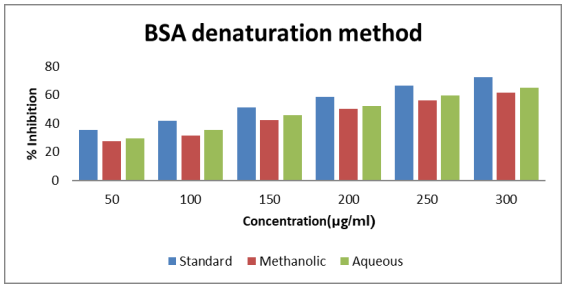


Fig 3: Effect of extract of *Zingiber officinale* leaves on BSA denaturation method

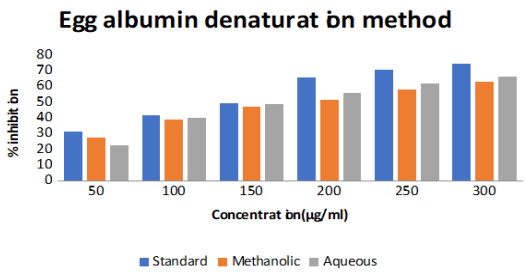


Fig 4: Effect of extract of *Zingiber officinale* leaves on egg albumin denaturation

In vitro anti-inflammatory activity of *Zingiber officinale*

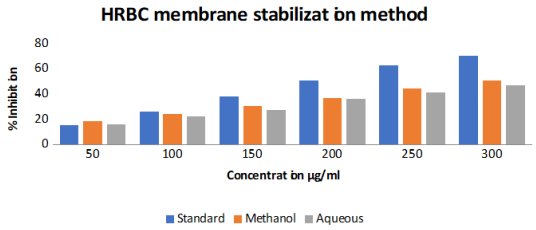


Fig 5: HRBC membrane stabilization test effect on Zingiber officinale leaves

flammatory diseases [26] The anti inflammatory activity of the leaves of ginger leaf extract was determined by inhibition of protein denaturation and membrane stabilization method[17]In anti-denaturation study, when heat is given activation of antigen and denaturation of protein occurs as a part of type III hypersensitivity reaction. The assay infers the ability of extract to stabilize the protein from denaturation. The absorbance of test sample with respect to control indicates the stabilization of membrane[2].

Lysosomes consist of activated constituents of neutrophils that can lead to tissue damage and inflammation. HRBC or erythrocyte membrane act similar to lysosomal membrane. In membrane stability assay, the ability to prevent the lysis of erythrocyte membrane infers the anti inflammatoryactivity[25].

Inflammation is one of the defense mechanisms against infections, tissue damage or injury. Non-steroidal anti inflammatoryagents(NSAIDs) are commonly used in the treatment of inflammation. Most NSAIDs act inhibiting the cyclooxygenase pathway. Main causes of inflammation in certain disease states such as arthritis, cancer and other inflammatory conditions are protein denaturation and lysosomal membrane lysis. One of the reasons for protein denaturation is production of auto antigens[26].

The leaf extract of ginger did show significant anti inflammatory activity may be due to the presence of shagaol and gingerol that act by suppressing the prostaglandin and leukotriene pathway by inhibiting the enzymes responsible for inflammation(27) or probably by inhibiting inducible nitric oxide synthase(iNOS) which is also a mediator of inflammation(28).

The % inhibition at 300µg/ml of aqueous extract, methanolic was observed at 64.88%, 61.30 % respectively compared to diclofenac which was 72.61% in Bovine Serum Albumin denaturation method, similarly at the same concentration, the aqueous and methanolic extract showed highest % of inhibition at 66.09% & 63.09% respectively in comparison with diclofenac which was 74.40% in egg albumin denaturation. In Haemolytic RBC membrane stabilization method, blood(1 ml of 10% RBC suspension) and Alsever's solution in equal quantities was gently mixed to inhibit haemolysis. Maximum inhibition of aqueous as well as methanolic extract was observed at 50.57% and 46.74 % respectively than standard Aspirin at 70.53 %, showing significant activity.

Conclusion:

The presence of number of bioactive constituents in the methanolic and aqueous leaf extract of zingiber officinale following GC-MS analysis indicated the usefulness of ginger leaves. The invitroanti inflammatory activity of zingiber officinale proved to have significant activity compared to the standard drugs.

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