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Antimicrobial susceptibility of selected medicinal fruit-*Myristica fragrans*

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ABSTRACT

Many countries across the world are interested in natural healing products. They are less toxic products. The genus Myristica fragrans belongs to the family Myristicaceae. The various parts of the plant have been used in traditional medicine for the treatment of inflammation, vomiting, asthma, hypertension, diarrhea, polyarthritis and gout etc. In the present study, the pericarp of Myristica fragrans is extracted successfully with solvents methanol, ethanol and hexane using Soxhlet apparatus. Phytochemical screenings were performed as per the standard procedures. Alkaloids, Saponins, Phytosterols, tannins, flavonoids and proteins were identified as the major compounds. The antibacterial activities of extracts were studied for various microorganisms by broth dilution method using streptomycin as the standard drug. The methanolic and ethanolic extract of M.fragrans pericarp showed remarkable activity against Staphylococcus aurous and Salmonella typhi respectively. Based on this TLC and Column chromatography were performed on the more bioactive methanol extract. Further investigation on the structure elucidation of the bioactive compound was done using UV, IR, and GC-MS analysis. The results of the present study suggest that the extracts of M.fragrans pericarp contains compounds with antibacterial properties that can be used as antimicrobial agents for the therapy of infectious diseases caused by pathogens.

Keywords: *Myristica fragrans*, Antibacterial activity, MIC, Phytochemicals, UV, IR, GCMS.

INTRODUCTION

For a long period of time, plants have been a valuable source of natural products for maintaining human health, especially in the last decade, with more intensive studies for natural therapies. Several plants were reported for their many therapeutic and pharmaceutical virtues, especially antioxidant, anti-tumor, and anti-infectious activities. A big part of the world's population still relies on the benefits of food for the treatment of common illnesses [1].These benefits are due to their big content of bioactive compounds [2]. Since the introduction of antibiotics there has been tremendous increase in the resistance of diverse bacterial pathogens [3, 4].This shift in susceptibility greatly affects the ability to successfully treat patients empirically. Plant derived products have been used for medicinal purposes for centuries.

At present, it is estimated that about 80% of the world population rely on botanical preparations as medicines to meet their health needs. Herbs and spices are generally considered safe and proved to be effective against certain ailments [5].They are also extensively used, particularly, in many Asian, African and other countries. In recent years, in view of their beneficial effects, use of spices/herbs has been gradually increasing in developed countries also.

Many plants have been used because of their antimicrobial traits, which are due to the secondary metabolites synthesized by the plants. A large number of phytochemicals belonging to several chemical classes have been shown to have inhibitory effects on all types of microorganisms in vitro [6]. These products are known by their active substances like tannins, alkaloids, carbohydrates, terpenoids, steroids and flavonoids etc. [7, 8]. Plant products have

been part of phytomedicines since time immemorial. This can be derived from barks, leaves, flowers, roots, fruits, seeds [9]. Knowledge of the chemical constituents of plants is desirable because such information will be value for synthesis of complex chemical substances [10, 11, 12].

Nutmeg (*Myristica fragrans*Houtt.) is believed to be a native of Banda Islands of Eastern Indonesia, formerly called the 'Spice Islands'. In India it is mainly cultivated in South India particularly in certain pockets of Kerala, Tamil Nadu and Karnataka [13]. The name 'Myristica' is derived from the Greek word 'Myron', a sweet liquid distilled from the plant[14].*M. fragrans* is an evergreen tree growing to a height of 18m. It belongs to the Myristicaceae family. Itproduces drupe type fruits, pyriform in shape, 6cm to 9cm long, yellowish skin with perpendicular groove around the fruit and whitish flesh. The flesh is about 1.3cm thick and contributes 75% to 85% of total weight. It splits when ripe revealing its red mace encasing its brown glossy seed. Nutmeg and mace are the two major primaryproducts of *M. fragrans* and are commerciallyconsidered as spices [15].

M.fragrans is getting attention as a new avenue in treating various diseases. The medicinal value of nutmeg in the treatment of several ailments ranging from nervous to digestive disorders [16] has been recognized worldwide since ancient times and it was regarded as a cure for plague. Recent studies relating to its anti-bacterial [17], anti-viral [18], anti-cancer [19], anti-proliferative [20], anti-oxidant [21], hepatoprotective [22], neuroprotective [23] and anti-obesity [24] effects reveal a wide scope for its application in the health sector.

So far, there is a lack of scientific reports that indicate the antimicrobial activities of the medicinal plant *M. fragrans*, especially for crude pericarp extracts of *M.fragrans*. Hence, this study was aimed at investigating the antimicrobial activities of methanolic, ethanolic and hexane extracts of pericarp of *M.fragrans* against both Gram-positive and Gram-negative bacteria.

MATERIALS AND METHODS

2.1 Collection of plant material

The fresh pericarp of *Myristica fragrans* fruits were collected from Lalam Village, Palai, Kottayam District, Kerala. It was then cut into small pieces and dried for 7 days at room temperature (25°C). The dried samples were ground into fine powder and kept away from heat, moisture, and sunlight.

2.2 Preparation of extract

The dried powdered sample of pericarp of *M. fragrans* was extracted using Soxhlet extraction method with methanol, ethanol and hexane solvents successively for 18 hours. Extracts were then filtered andconcentrated under reduced pressure by using a rotary evaporator. The crude extracts were then stored in refrigerator for use in further studies.

2.3 Phytochemical Analysis

The extracts were subjected to phytochemical screening for the presence of alkaloids, carbohydrates, glycosides, tannins, terpenes, saponins, phenols, flavonoids, triterpenes, terpenoids, quinones, proteins & amino acids. Phytochemical screenings of the extracts were carried out as per standard methods [25].

2.4 Test for antibacterial activity

2.4.1 Bacterial strains

Antibacterial activities of the extracts were tested against certain gram-negative bacteria (*Escherichia coli, Klebsiella pneumoniae, Pseudomonas Aeruginosa , Salmonella typhi, Proteus mirabilis, Proteus vulgaris)* and one gram-positive bacteria (*Staphylococcus aurous)* using broth dilution method.

2.4.2 Well Diffusion method

The *in vitro* antibacterial activities of the extracts were tested against the bacterial species. One day prior to the experiment, the bacterial cultures were inoculated in broth and incubated overnight at 37°C. Inoculation medium containing 24 hr grown culture was added aseptically to the nutrient medium and mixed thoroughly to get the uniform distribution. This solution was poured (25 mL in each dish) into petri dishes and then allowed to attain room temperature. Wells (6 mm in diameter) were cut in the agar plates using proper sterile tubes. Then, wells were filled up to the surface of agar with 0.1 mL of the test compounds dissolved in DMSO (200 µM/mL). The plates were allowed to stand for an hour in order to facilitate the diffusion of the drug solution. Then the plates were incubated at37°C for 24 hr forbacteria and the diameters of the inhibition zones were read. Minimum inhibitory concentrations (MICs) were determined by using serial dilution method. The lowest concentration (µg/mL) of compound, which inhibits the growth of bacteria after 24 hr incubation at 37°C, was taken as the MIC. The concentration of DMSO in the medium did not affect the growth of any of the microorganisms tested.

2.5Chromatographic Analysis

2.5.1 Thin layer chromatography (TLC)

TLC experiments were performed on TLC pre-coated silica gel (60-120 μm mesh size) plates using an appropriate solvent system.

2.5.2 Column Chromatography

2.5.2.1 Preparation of methanolextract for Column:

The methanolextract (15g) was taken in a china dish and minimum quantity of activated (heated for 30 min at 110^0C in hot air oven) silica gel (60-120#) was added to it to get uniform consistency. It was air dried and larger lumps were broken to get uniform particle size.

2.5.2.2 Fractionation and isolation:

Column chromatography was used to further isolate some compounds of the mixture. Columns were packed with silica gel (60-120μm mesh size) using a wet method. The extract was fractionated by column chromatography using petroleum ether: ethyl acetate gradient (10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% and 100% ethyl acetate). It was followed by chloroform in ethyl acetate (10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% and 100% chloroform). The fractions were collected, concentrated and then monitored with TLCand spotswere detected by ultraviolet illumination (254 and 365mm) and iodine stream. The fractions which showed the same profile of chromatograms were combined together. The combined fractions were placed on the air current to facilitate drying. All the resultant fractions were subjected to Spectroscopic analysis.

2.6 Spectroscopic Analysis

2.6.1 UV/VIS Spectroscopic Analysis

UV-visiblespectroscopy uses light in the visible ranges or its adjacent ranges i.e. near ultraviolet(UV) and near infrared (NIR) ranges. The colour of the chemicals involved is directlyaffects the absorption in the visible ranges. Molecules undergo electronic transitions inthese ranges of the electromagnetic spectrum.

2.6.2 FT-IR Spectroscopic Analysis

In the molecular diagnosis of vibrational frequencies of absorption bands it is extremely useful to refer to the illustrated values of various functional groups and their associated characteristic group of frequencies ranges. In order to give conclusive ideas about the structure of the compound under investigation, it is necessary to have an assignment for the IR-adsorption bands corresponding to the active group in the compound.

2.6.3 GC MS Analysis of methanol fraction of *M.fragrans* extract

GC-MS technique was used in this study to identify the components present in the pericarp extract of the test plant. GC-MS technique was carried out at The *Cashew Export Promotion Council* (*CEPC*), Kollam, Kerala. GC-MS analysis was carried out on a GC clarus 500 Varian, USA system comprising a AOC-20I auto sampler and gas chromatograph interfaced to a mass spectrophotometer instrument employing the following conditions: Column Elite-1 fused silica capillary column (30mm×0.25mm I.D ×1 μ M df, composed of 100% Dimethyl poly siloxane), operating in electron impact mode at 70 eV; helium (99.999%) was used as carrier gas at a constant flow of 1ml/min and an injection volume of 0.5 μ l was employed (split ratio of10:1) injector temperature 250 °C; ion-source temperature 280 °C. The oven temperature was programmed from 110 °C (isothermal for 2 min), with an increase of 10 °C/min, to 200 °C, then 5 °C/min to 280 °C, ending with a 9 min isothermal at 280 °C. Mass spectra were taken at 70 eV; a scan interval of 0.5 seconds and fragments from 45 to 450 Da. Total GC running time is 46min.

RESULT AND DISCUSSION

3.1 Phytochemical testing

The results of qualitative screening of phytochemical components in methanol, ethanol and hexane extracts of *M.fragrans* fruits revealed the presence of alkaloids, carbohydrates, glycosides, tannins, terpenes, saponins, phenols, flavonoids, triterpenes, terpenoids, quinones, proteins & amino acids presented in Table1. The methanol extract of *M.fragrans* contains alkaloids, carbohydrates, saponins, glycosides, terpenoids, flavonoids, proteins, triterpenes and quinones. The ethanol extract contains alkaloids,proteins and tannins only. The hexane extract contains alkaloids, carbohydrates, saponins, terpenoids, flavonoids, tannins and quinones.

3.2 Antibacterial testing

Methanol, ethanol and hexane extracts of *M.Fragrans* fruits were tested against various gram negative and gram positive bacteriapresented in (table- 2).The methanol and ethanol extracts of *M.Fragrans* pericarp were shown remarkable activity against*staphylococcus aurous* and*Salmonella typhi* respectively.

Table-1. The phytochemical constituents in the pericarp extract of *M.Fragrans* fruits

Phytochemicals	Hexane	Ethanol	Methanol
Alkaloids (Hager's Test)	+	+	+
Carbohydrate(Fehling Test)	+	_	+
Glycosides(Borntragers Test)	_	_	+
Saponins (Froth Test & Foam Test)	+	_	+
Proteins (Xanthoproteic Test)	_	+	+
Phenols(Ferric Chloride Test)	_	_	+
Flavonoids(Lead Acetate Test)	+	_	+
Triterpenes	_	_	+
Terpenoids (Salkowskis test)	+	_	+
Tannins(Braymers Test)	_	+	+
Quinones	+	_	+

Table -2.MICs in µg /ml values of various extracts of *M.Fragrans.*

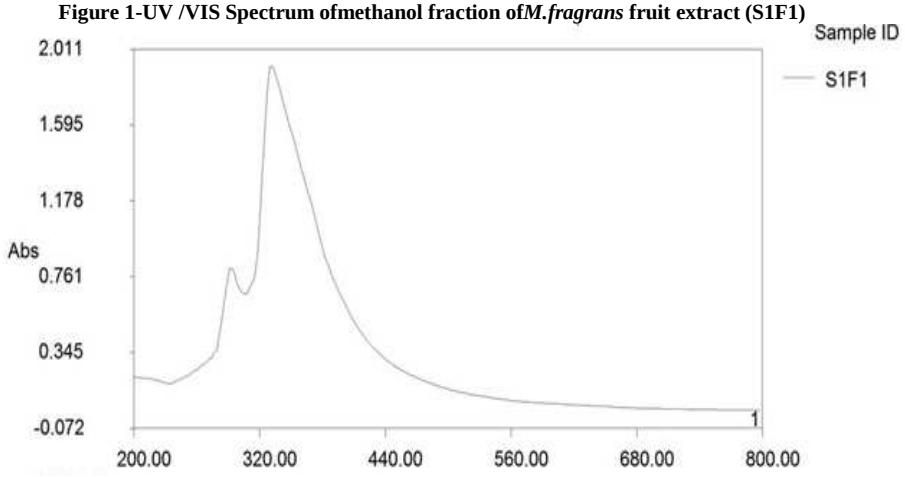
Microorganisms MIC(microgram/mL)	Methanol extract	Ethanol extract	Hexane extract
Staphylococcus aurous	28	86	72
Klebsiella	34	54	80
Pseudomonas	46	40	74
Escherichia Coli	80	102	44
Salmonella typhi	42	30	64
Proteus mirabilis	60	52	88
Proteus vulgaris	80	69	86
Streptomycin	4	6	5

3.3 Spectroscopic analysis

3.3.1 UV spectrum

UV-visible spectroscopy or in other words ultraviolet-visible spectrophotometry (UV-Vis) related to the spectroscopy of photons in the UV-visible region. In UV-visible spectroscopy the absorption measures transitions from the ground state to the excited state. [26].

Figure 1-UV /VIS Spectrum ofmethanol fraction of*M.fragrans* fruit extract (S1F1)



The peak at 332.0 and at 291.2 indicates that the extract may contain the functional groups C═C and C═O respectively.

3.3.2 FT-IR Spectroscopic Analysis of *M.fragrans*

The Fourier transform infrared spectrum was recorded for the sample in the range 450 cm-1 to 4000 cm-1 using the instrument FT-IR 4100 type A spectrometer. The functional groups are assigned from the FTIR spectrum (Figure -2) as follows. The peak at 3412.68 cm-1, which indicates the presence of phenolic group, corresponds to O-H stretching vibrations, it may be –COOH group. The stretching vibrations of C-H group is found corresponding to the peak at 3004.77cm-1. The peak at 2141.07 cm-1 corresponds to C≡C stretching or C≡N stretching vibrations. The strong and well defined peak at 1704.17 cm-1 is due to C═O stretching. The peak at 1421.02 cm-1 is attributed to O-H bending (deformation) or C─H bending vibrations. The peaks at 1360.14, 1222.31, 1092.82 cm-1 corresponds to C─N stretching, C─C stretching and C─O stretching respectively.

Figure-2- FT-IR Spectrum of methanol fraction of *M.fragrans* fruit extracts (S1F1)

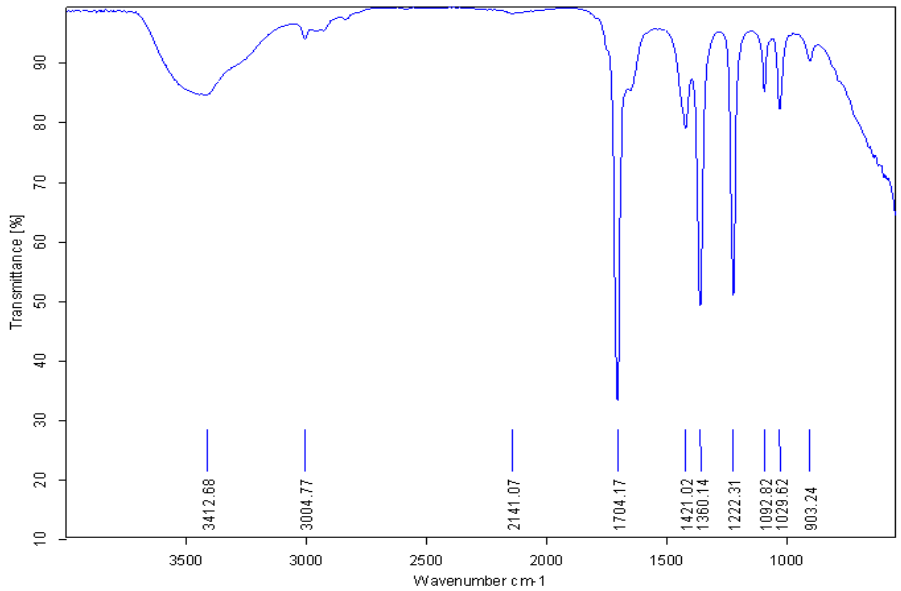


Table-4 Functional groups present methanol fraction of *M.fragrans* fruit extract (S1F1)

Sl.No.	Wave number (cm-1)	Spectroscopic Assignments
1	3412.68	O─H stretching. Inter molecular hydrogen bonded.(may be –COOH group)
2	3004.77	C─H stretching
3	2141.07	C≡C stretching or C≡N stretching
4	1704.17	C═O stretching (strong, well defined)
5	1421.02	C─H bending ,O─H bending (deformation)
6	1360.14	C─N stretching
7	1222.31	C─C stretching
8	1092.82	C─O stretching

3.3.3 GCMS Analysis

Chromatogram obtained by GCMS Analysis of *M.fragrans* fruit extract is given below.

Figure-3.Chromatogram obtained by GCMS Analysis of *M.fragrans* fruit

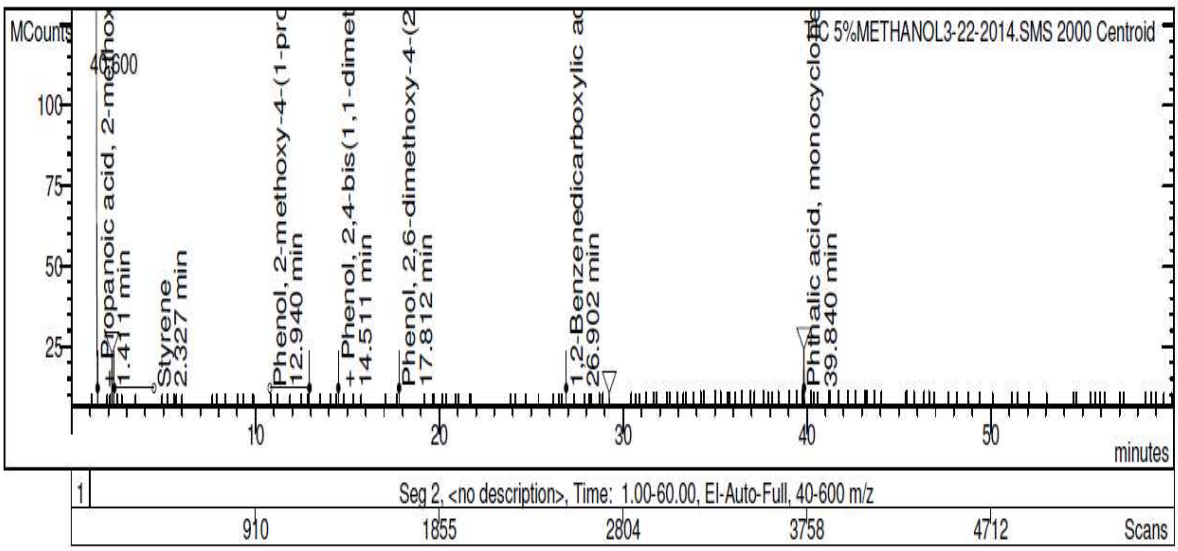
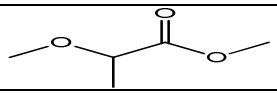
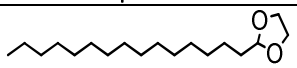
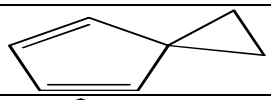
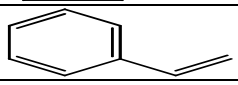
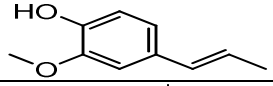
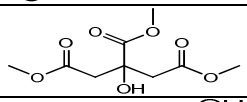
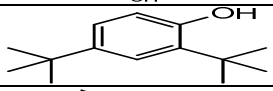
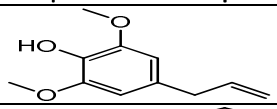
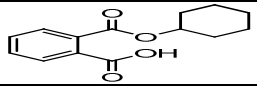


Table-5.Chemical composition of *M.fragrans* by GC-MS analysis

Sl. No.	RT	Compound Name	Structure	Mol.Wt.	Mol. Formula	Peak Area(%)
1.	1.411	Propanoic acid, 2-methoxy-, methyl ester		118.06	$C_5H_{10}O_3$	3.45
2	1.495	2-pentadecyl-1,3-dioxolane		284.48	$C_{18}H_{36}O_2$	1.15
3	1.766	Spiro[2.4]hepta-4,6-diene		92.14	C_7H_8	1.47
4	2.327	Styrene		104.06	C_8H_8	0.15
5	12.940	(*E*)-2-methoxy-4-(prop-1-enyl)phenol		164.20	$C_{10}H_{12}O_2$	0.35
6	13.176	trimethyl 2-hydroxypropane-1,2,3-tricarboxylate		234.20	$C_9H_{14}O_7$	0.28
7	14.511	2,4-di-*tert*-butylphenol		206.32	$C_{14}H_{22}O$	0.62
8	17.812	4-allyl-2,6-dimethoxyphenol		194.23	$C_{11}H_{14}O_3$	1.44
9	39.840	2-(cyclohexyloxycarbonyl)benzoic acid		248.27	$C_{14}H_{16}O_4$	2.48

Nine compounds were identified in methanol extract fraction of *M.fragrans* fruit by GC-MS analysis. The chromatogram obtained by methanol fraction of *M.fragrans* fruit was shown in Fig.3.The active principle, area of the peak, Retention Time (RT), Molecular formula and Molecular weight were presented in Table 5. The prevailing compounds were Propanoic acid,2-methoxy-,methylester(3.45%), 2-(cyclohexyloxycarbonyl)benzoic acid (2.48%), 4-allyl-2,6-dimethoxy phenol(1.44%), Spiro[2,4]hepta-4,6-diene(1.47%), 2-pentadecyl-1,3-dioxolane(1.15%), 2,4-di-*tert*-butylphenol(0.62%), (*E*)-2-methoxy-4-(prop-1-enyl)phenol(0.35%),trimethyl,2-hydroxypropane-1,2,3-tricarboxylate (0.28%),Styrene(0.15%).

CONCLUSION

Extraction of bioactive compounds from medicinal plants facilitates pharmacology studies leading to synthesis of more potent drug for meeting demand for effective and safe use. The results of the present study suggest that the extract of *M.fragrans* contains compounds with antibacterial property that can be used as antimicrobial agents for the therapy of infectious diseases caused by pathogens. The results form a good basis for the selection of plant species for future phytochemical and pharmacological investigation.

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