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BIOACTIVITY OF *Myristica fragrans* METHANOL EXTRACT

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ABSTRACT

Objectives: *Myristica fragrans*, commonly called nutmeg, is an aromatic evergreen tree. It has been found to possess medical value and has been used to treat rheumatism and stomach complaints in Indonesia, Malaysia, England, and China, because of its essential oil. However, the effect of *Myristica fragrans* methanol extracts on the area of cytotoxicity is unexplored, hence the present work was aimed at evaluating the effect of extracts on cancer cell line and also on clinical pathogens. Methods: Phytochemical and analytical studies (TLC and GC-MS) was evaluated with methanol extract of *Myristica fragrans*. Antimicrobial activity of *Myristica fragrans* was also checked against nine clinical pathogens. Antioxidant and anticancer study against MG 63 cell line was also carried out. Result: Results has shown that *Myristica fragrans* extract posses antimicrobial activity against both Gram positive and Gram negative organisms and moderate antioxidant and anticancer activity. Conclusion: The present support the fact that *Myristica fragrans* extract can be used as future drug.

KEY WORDS: Nutmeg, Phytochemical studies, Analytical studies, Clinical pathogens, MG 63 cell line.

INTRODUCTION

Medicinal plants have been used to cure disease and maintain health from time memorial. However, the search for bioactive compounds in these plants has intensified for the past

decades. Phenolic compounds have been demonstrated to have anticancer, antioxidant, antimicrobial activity and promote immunological functions [1]. *Myristica fragrans* Houtt (nutmeg) is one of the plants commonly found in Asian medicinal ingredients. It is composed of skin, flesh, seed, and mace. Nutmeg is the seed kernel inside the fruit and mace is the fleshy red, net-like skin covering (aril) on the kernel [2]. The main constituents of *Myristica fragrans* have been found to be alkyl benzene derivatives (myristicin, elemicin, safrole, etc.), terpenes, alpha-pinene, beta-pinene, myristic acid, trimyristin [3, 4, 5], neolignan (myrislignan), and macelignan [6].

The chemical constituents of *Myristica fragrans* have been investigated by scientists from various disciplines for hypolipidemic and hypocholesterolemic effects, antimicrobial, antidepressant, aphrodisiac, memory-enhancing, antioxidant, and hepatoprotective properties[2]. In traditional medicine, the seed kernel (nutmeg) is widely used as carminative, astringent, hypolipidaemic, antithrombotic, antiplatelet aggregation, antifungal, aphrodisiac[7], treating flatulence, nausea, and dyspepsia [8]. Mace is widely used as flavouring agent, hair dye, and in folk medicine for other remedies. It also possesses antipapillomagenic, anticarcinogenic [9], and anti-inflammatory activities [10].

In dentistry application, macelignan, an active compound from seed has been found to have strong anticarcinogenic activity, along with antibacterial effect against oral microorganisms' such as *Streptococcus* sp., and *Lactobacillus* sp., and exhibited weak activity for *Actinomyces viscosus*, *Porphyromonas gingivalis* and *Staphylococcus aureus* [6]. Narasimhan and Dhake [11] (2006) reported that trimyristin, an active compound obtained from seed of *Myristica fragrans*, also exhibited good antibacterial properties against Gram-positive and Gram negative bacteria.

In this study methanol extract of *Myristica fragrans* was evaluated for antimicrobial, antioxidant and anticancer effects against osteocarcoma by experimental approaches.

MATERIALS AND METHODS

Chemicals

All chemicals and solvents used in this study were of high purity and analytical grade.

Sample extraction

The *Myristica fragrans* were collected dried and powdered. Powdered sample were extracted with methanol using soxhlet apparatus. The extracted solvent is later collected, dried and stored for further studies.

Phytochemical studies

Phytochemical studies were done with methanol extract of plant leaves to primarily detect the presence of various compounds.

Detection of alkaloids

Solvent free extract, 5 mg was stirred with few ml of diluted hydrochloric acid and filtered. The filtrate was tested carefully with various alkaloidal reagents [12].

A. Hager's Test: Filtrates were treated with Hager's reagent (saturated picric acid solution). Presence of alkaloids was confirmed by the formation of yellow coloured precipitate.

B. Wagner's Test: To two ml of filtrate, few drops of Wagner's reagent were added along the side of test tube. A reddish brown precipitates indicated positive test [13].

Wagner's reagent: Iodine (1.27g) and potassium iodide (0.92 g) was dissolved in 5 ml of water and made up to 100 ml with distilled water.

Detection of carbohydrates and glycosides

5 mg of extract was dissolved in 5 ml of water and filtered. The filtrate was subjected to the following tests [14].

A. Fehling's Test: 1 ml of filtrate was boiled on water bath with 1 ml of each of Fehling's solutions A and B. Appearance of red precipitate confirmed the presence of sugar.

Fehling's solution A: Copper sulphate (34.66 g) was dissolved in distilled water and made up to 500 ml using distilled water.

Fehling's solution B: Potassium sodium tartarate (173 g) and sodium hydroxide (50g) was dissolved in water and made up to 500 ml.

B. Molish Test: To 2 ml of filtrate, two drops of alcoholic solution of α napthol were added, the mixture was shaken well and 1 ml of conc. sulphuric acid was added slowly along the sides of test tube and allowed to stand. Formation of violet ring indicated the presence of carbohydrates.

Detection of phytosterols

Libermann Burchard's Test: The extract (5 mg) was dissolved in 2 ml acetic anhydride. To this, one or two drops of conc. sulphuric acid were added slowly along the sides of the test tube. An array of colour changes indicated the presence of phytosterols [15].

Detection of phenolic compounds

Ferric chloride test: The extract (2 mg) was dissolved in 5 ml of distilled water. To this, few drops of neutral 5% ferric chloride solution was added. Appearance of green colour indicates the presence of phenolic compounds [16].

Detection of flavonoids

Alkaline Reagent Test: Extracts were treated with few drops of sodium hydroxide solution. Formation of intense yellow colour, which becomes colourless on addition of dilute acid, which indicated the presence of flavonoids.

Lead acetate Test: One ml of the plant extract was added in a test tube. To this 1ml of 5% lead acetate and the mixture was allowed to stand for few minutes. The formation of precipitates in samples confirmed the presence of flavonoids.

Detection of Tannins

About 0.5 g of extract was stirred with about 10 ml of distilled water and then filtered. Few drops of 1% ferric chloride solution were added to 2 ml of the filtrate. Formation of a bluish black, bluish green or green precipitate confirmed the presence of tannins.

Detection of terpenoids (Salkowski test)

Two ml of chloroform was added to the extract. Concentrated H_2SO_4 (3 ml) was carefully added to form a layer. Appearance of reddish brown colouration on the interface indicates the presence of terpenoids.

Analytical methods

Fractionation of the crude extract using TLC

Using pre-coated TLC F254 plates, the crude extract was fractionated using different combinations of hexane/chloroform/methanol solvents (2:1:1) and methanol/hexane (3:2) as the mobile phase. Separated components were viewed in visible light, under UV at 360 nm, by fluorescence quenching less than 254 nm. Separation done with mobile phase of hexane: chloroform: methanol was fractionated and recorded.

Gas Chromatography Mass Spectrometry

Methanol extract of *Myristica fragrans* were analyzed by GC-MS. Perkin Elmer Clarus 680 gas chromatographic instrument equipped with a mass spectrometer detector (Clarus 600 model) and an Elite-5MS (30.0 m, 0.25 mmID, 250 µm df) column was used. The carrier gas used was helium at a flow rate of 1 ml min^{-1}. The following temperature program was used: initially the oven temperature was held at 60°C for 2 min and then ramped from 10°C/min to 300°C with hold time for 4 min, total run time 30 min. The temperature of the injector was maintained at 300°C. The ion trap was operated at 70 eV with a scan range of m/z from 50 to 600. A sample of 1 µl was injected in split mode (10:1). The intermediate and end product was identified based on the Wiley registry of mass spectral data.

Antimicrobial study

The antibacterial activity of methanol extract of *Myristica fragrans* against nine bacterial pathogens was evaluated by using agar well diffusion method [17, 18]. Muller Hinton Agar (MHA) plates were inoculated with selected bacterium. Wells of 8 mm size were made with sterile borer on agar plates. Four different volumes (25 µl, 50µl, 75 µl, 100 µl) of the plant extract were poured into each well of inoculated plates. Respective solvent for particular solvent extracts was used as a negative control. Then they were left at room temperature for ten minutes allowing the diffusion of the plant extract into the agar [19]. After incubation for 24 hrs at 37^{o}C, the plates were observed for clear zone. Antibacterial activity of the extract was identified by an inhibition zone surrounding the well containing the plant extract. The zone of inhibition was measured and expressed in millimeters (mm). Clear zone of the plant extract and comparison with negative control was recorded [20].

Antioxidant Study

The antioxidant activity of the methanol extract was evaluated by DPPH radical scavenging assay which was originally described by Blois [21]. DPPH (2, 2-diphenyl-1-picrylhydrazyl) is a synthetic free radical with deep violet colour when is in form of solution which has a λ_{max} at 517nm. It can accept an electron or hydrogen radical to become stable diamagnetic molecule and appear as light purple in colour which indicates the scavenging of DPPH and the substance has antioxidant activity.

Methanol solutions were prepared with all the three extracts. Methanol solution of DPPH was used as negative control. 500µl of each sample and 500 µl of DPPH solution was allowed to react and incubated at room temperature for 30mins under dark conditions. Absorbance was

taken at λ_{max} i.e. 517nm against a blank which was 500µl of methanol. Percentage inhibition was calculated by the following equation to conclude the presence of antioxidant activity of the extracts.

Percentage of inhibition = (OD control – OD sample / OD control) x 100

Anticancer study

Cell line

The human osteosarcoma cell line (MG 63) was obtained from National Centre for Cell Science (NCCS), Pune and grown in Eagles Minimum Essential Medium containing 10% fetal bovine serum (FBS). The cells were maintained at 37°C, 5% CO_2, 95% air and 100% relative humidity. Maintenance cultures were passaged weekly, and the culture medium was changed twice a week.

Cell treatment procedure

The monolayer cells were detached with trypsin-ethylenediaminetetraacetic acid (EDTA) to make single cell suspensions and viable cells were counted using a hemocytometer and diluted with medium containing 5% FBS to give final density of $1x10^5$ cells/ml. One hundred microlitres per well of cell suspension were seeded into 96-well plates at plating density of 10,000 cells/well and incubated to allow for cell attachment at 37°C, 5% CO_2, 95% air and 100% relative humidity. After 24 h the cells were treated with serial concentrations of the test samples. They were initially dissolved in neat dimethylsulfoxide (DMSO) and an aliquot of the sample solution was diluted to twice the desired final maximum test concentration with serum free medium. Additional four serial dilutions were made to provide a total of five sample concentrations. Aliquots of 100 µl of these different sample dilutions were added to the appropriate wells already containing 100 µl of medium, resulting in the required final sample concentrations. Following sample addition, the plates were incubated for an additional 48 h at 37°C, 5% CO_2, 95% air and 100% relative humidity. The medium containing without samples were served as control and triplicate was maintained for all concentrations.

MTT assay

After 48 h of incubation, 15µl of MTT (5mg/ml) in phosphate buffered saline (PBS) was added to each well and incubated at 37°C for 4h. The medium with MTT was then flicked off and the formed formazan crystals were solubilized in 100µl of DMSO and then measured the absorbance at 570 nm using micro plate reader.

The percentage of cell viability was then calculated with respect to control as follows:

Percentage of cell viability = [A] Test / [A]control x 100

The percentage of cell inhibition was determined using the following formula:

Percentage of cell inhibition = 100- Abs (sample)/Abs (control) x100.

Nonlinear regression graph was plotted between percentage of cell inhibition and Log concentration and IC50 was determined using GraphPad Prism software [22, 23].

RESULTS

Phytochemical study

In recent years, secondary plant metabolites are being extensively investigated as a source of medicinal agents. It has been accepted that natural compounds play an important role in health care. Result of phytochemical studies of *Myristica fragrans* extract with methanol is presented in a table form in table 1. According to the result, alkaloids, steroids, glycosides and phenols are present in *Myristica fragrans*. However Fehling's and Molish test for carbohydrate and Salkowski test for terpenoids showed negative response. According to Singh et al.[24] phenolic compounds possess biological properties such as antiapoptosis, antiaging, anticarcinogen, antiinflammation, as well as inhibition of angiogenesis and cell proliferation activities. Phytosterol acts as growth hormones in plants. The plant has medicinal property due to presence of these phytochemicals [25].

Table 1: Phytochemical study of *Myristica fragrans* methanol extract

Sl no	Phytochemical test	Result
1	Alkaloids	
	Hager's test	+
	Wagner's test	+
2	Carbohydrates	
	Fehling's test	-
	Molish Test	-
3	Phytosterols	
	Libermann Burchard's Test	+
4	Phenols	
	Ferric chloride test	+
5	Flavonoids	
	Alkaline Reagent Test	-
	Lead acetate Test:	-
6	Tannin	-
	Terpenoids	-
7	Salkowski test	-

Analytical studies

TLC

TLC was done to fractionate each components of the extract by its characteristic Rf values. Separated spots were observed under UV light (fig.1). Two separated spots have been observed under UV light for methanol extract of *Myristica fragrans*. Separated spots on TLC plates indicate presence of different compounds which were further analyzed by GC-MS.

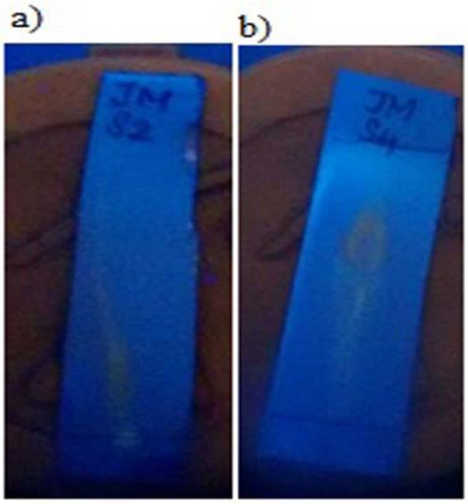

Figure 1: Separated spots on the TLC plate visualized under UV light, a) solvent 1 and b) solvent 2.

GC-MS

In order to determine the compounds present in the extract of *Myristica fragrans*, GC-MS analysis was done. This analysis revealed that methanol extract *Myristica fragrans* contain different compounds. Some of them are known for their biological activity whereas activity of a few compounds remains unknown. The GC-MS chromatogram of methanol extract is presented in fig.2. From GC-MS analysis, it has been seen that 3-cyclohexen-1-ol, 4-methyl-1-(1-methylethyl is present in methanol extract of *Myristica fragrans*, which is reported to be a derivative of limonene [26] which possess antimicrobial, and antiseptic activity [27]. Benzene, 1,2- dimethoxy-4-(2- propyl) is known as methyl eugenol which is widely used as insecticide. N-hexadecanoic acid (palmitic acid), found in methanol extract of *Myristica fragrans* which posses antibacterial and cholesterolaemic effects, selective toxicity to human leukemic cells. It also has shown in vivo antitumor activity in mice by making a target to DNA topoisomerase I. 2-hydroxy-4-4isopropyl-7-methoxytropone is reported to possess anticancer activity. Activities of these compounds are believed to be responsible for the activities shown by *Myristica fragrans* extracts.

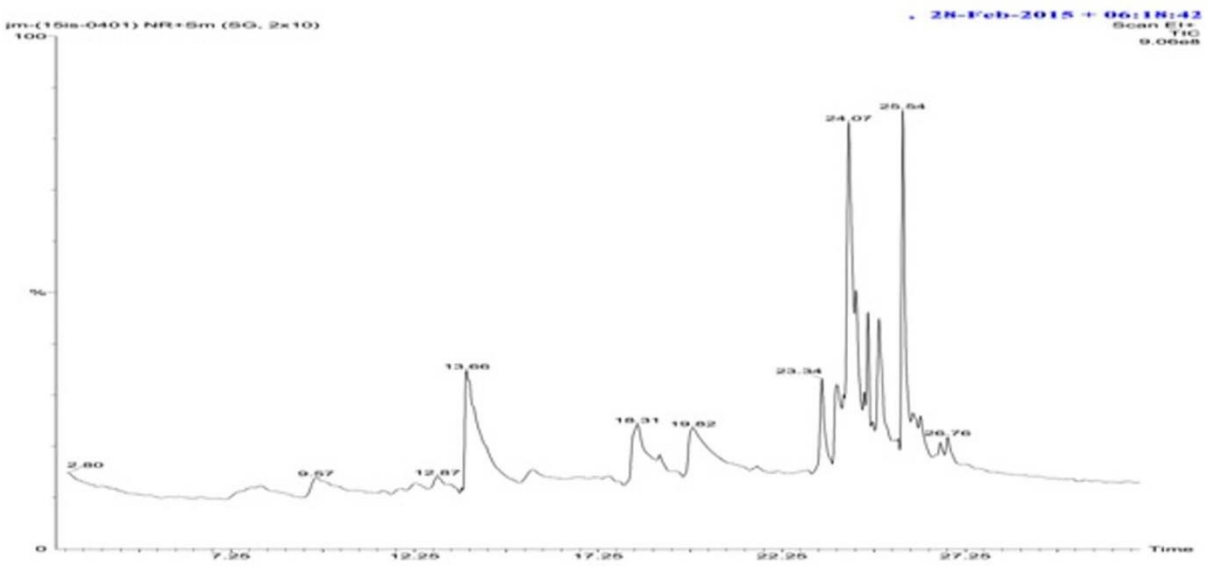

Figure 2: GC-MS chromatogram of *Myristica fragrans* methanol extract.

Antimicrobial Test

Antimicrobial activity of *Myristica fragrans* was checked against nine clinical pathogens. Antimicrobial activity was determined by measuring zone of inhibition formed after incubation period. The methanol extract showed characteristic zone of inhibition against five pathogens including *Pseudomonas aeruginosa*, *Serretia* sp., *Salmonella* sp. and *Klebsiella* sp. and *Escherichia coli* among nine test pathogens. The zone of inhibition was very pronounced. Highest zone of inhibition was observed at 100mg/ml concentration against *Pseudomonas aeruginosa*. Methanol extract of *Myristica fragrans* showed antimicrobial activity against both Gram positive and Gram negative organisms. Table 2 indicates the result of the antimicrobial activities of methanol extract of *Myristica fragrans*.

Table 2: Antimicrobial activity of *Myristica fragrans* methanol extract against clinical pathogens

Sl no	Organisms	Zone of inhibition (mm)			
		25mg/ml	50mg/ml	75mg/ml	100mg/ml
1	*Pseudomonas aeruginosa*	19	20	22	22
2	*Shigella* sp.	-	-	-	-
3	*Serretia* sp.	7	9	13	16
4	*Salmonella* sp.	-	-	-	-
5	*Klebsiella* sp.	5	5	6	7
6	*Enterobacter* sp.	-	-	-	-
7	*Proteus mirabilis*	-	-	-	-
8	*Staphylococcus* sp.	8	8	9	9
9	*Escherichia coli*	9	9	10	12

Antioxidant Test

The antioxidant activity of the extracts was evaluated by DPPH radical scavenging assay which was originally described by Blois [21]. DPPH (2, 2-diphenyl-1-picrylhydrazyl) is a synthetic free radical with deep violet colour when is in form of solution which has a λ_{max} at 517nm. Methanol extract of *Myristica fragrans*, methanol extract has shown moderate antioxidant (table 3). The reducing power of methanol extract indicates presence of some compounds in *Myristica fragrans* extracts which can donate electron and could react with free radicals to convert them into more stable products and to terminate radical chain reactions. Increased absorbance of reaction mixture indicates increased reducing power of the extract [28].

Table 3: Antioxidant result of *Myristica fragrans* methanol extract.

Sample	OD Sample At 517nm	Percentage Of Inhibition
Standard	0.823	
Methanol extract	0.225	72%

Anticancer study

Anticancer study of the *Myristica fragrans* was done against human osteosarcoma cell line (MG 63). Anticancer activity of methanol extract was checked. The activity of methanol extract was shown in fig 3. In methanol extract cell viability is decreased with increased concentration of extract, which indicates the moderate activity of the extract. On the other hand,12.5 µg/ml concentration of extract showed less than 5% of cell inhibition while at 50 µg/ml concentration of extract, percentage of cell inhibition reaches 63%, which indicates that percentage of cell inhibition is increased with increasing concentration of extract [29] . The IC 50 value is calculated to be 40.39 µg/ml at which concentration the percentage of cell inhibition reaches 50. The data indicates the presence of anticancer activity of both methanol extract of *Myristica fragrans*.

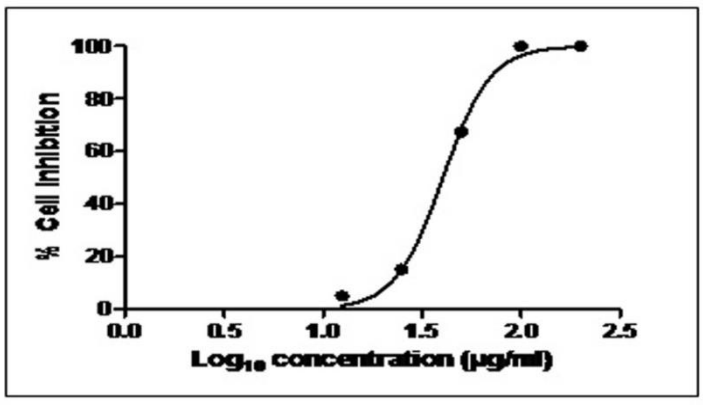

Figure 3: Anticancer activity of *Myristica fragrans* methanol extract.

CONCLUSION

Based on the current finding, it is proved that the methanol extract of *Myristica fragrans* exhibited good potential as antimicrobial, antioxidant and anticancer agents. Methanol extract showed antimicrobial activity against both Gram positive and Gram negative organisms. Thus *Myristica fragrans* extract could be considered as an alternate drug. In addition, more research is needed to isolate and identify the bioactive compounds from *Myristica fragrans* extract and their ability to serve as drug in near future.

There is no conflict of interest.

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