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Phytochemical Analysis and Anticancer Activity of Essential Oil from *Myristica fragrans*

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Abstract:

Volatile oil from the leaves of *Myristica fragrans* was isolated and characterized by Gas Chromatography- Mass Spectroscopy. Twenty constituents from 20 peaks were identified beta- pinene (22.69%), alpha-pinene (14.06%), alpha-thujen (13.93%) and p-menth 1-en-4-ol (10.53%) are as the major constituents and this leaf oil contains the minor constituents like camphene (3.92%), α-terpinene (3.42%), Limonene (9.56%) p-cymene (6.86%) Linalool (7.41%), beta myrcen (4.81%), alpha fellandrene (3.14%), 3-carene (3.54%), allyl catechol methylene (8.32%), myristicin (7.20%), elermicin (9.85%), beta ocimene (4.74%), gamma terpinene (4.75%), alpha terpinolene (4.38%), p-menth 2-en-1-ol (3.30%) and α-terpineol (6.00%). The antimicrobial activity of oil was tested against human and plant pathogenic bacteria and fungi. The oil showed significant inhibitory activity against the bacteria, *Enterococcus faecalis* (1.3cm), *Lactococcus plantarum* (0.9cm) and *Proteus vulgaris* (0.6cm) and the fungus *Candida tropicalis* (1.3cm), *Candida albicans* (0.8cm), *Rhizomucor miehei* (0.6cm) and *Candida glabrata* (0.6cm). No inhibitiory activity was observed against the bacteria *Clostridium pertringens*, *Klebsiella Pneumoniae* and *Bacillus megaterium*. There is no inhibitory activity of oil against the fungi, *Aspergillus niger and Aspergillus fumigates.* Using fluorescent stains localize cortex region, phloem fibres, oil ducts, phenol secreting cells and vascular cambial cells. *Myristica fragrans* showed 100% larvicide activity. The addition of various concentrations of essential oil of *Myristica fragrans* in the MCF-7 breast cancer cell line and A-357 epidermal skin cancer cell line showed cytotoxic activity.

Key Words: *Myristica fragrans*, Antimicrobial Screening, GC/MS Analysis, Histochemistry, Anticancer, Larvicide.

Introduction

Medicinal plants have been used for centuries as remedies for human diseases, because they contain components as therapeutic value. Historically many plant oils and extracts, such as

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ginger, garlic, curcuma, tea tree, myrrh and clove have been reported to have antimicrobial properties[1,2].

Myristica fragrans is a perennial edible plant of the Annanceae family is a berry that grows wild in the evergreen forests of West Africa[3]. The seeds are economically and medicinally important[4]. The kernel obtained from the seeds is a popular condiment used as a spicing agent. The seeds are embedded in a white sweet-smelling pulp and are most economically important part of the tree. They are aromatic and are used after grinding to a powder as a condiment in food providing flavour resembling that of nutmeg (*Myristica fragrans*).

They are also used as an aromatic stimulating addition to medicine and snuff. The nutmeg seed is one of four components of the fruit obtained from the nutmeg tree, *Myristica frangans Houtt (Myristicaceae).* About 30-55% of the seed consists of oils and 45-60% consists of solid matter including cellulose materials. There are two types of oils: The "essential oil of nutmeg" also called the "volatile oil" accounts for 5-15% of the nutmeg seed and the "fixed oil of nutmeg" sometimes called "nutmeg butter" or expressed oil of nutmeg accounts for 24-40% of the nutmeg seed. The relative percentages of the different components will vary depending on the geographical origin of the nutmeg. In the present study certain works such as phytochemical characterization, antimicrobial activity of oil, histochemical studies, cytotoxic effects of essential oil extract against MCF-7 ad A375 cell line.

Materials and Methods:

Plant Material:

The leaves of *Myristica fragrans* was collected from the western Ghats of Tamil Nadu (Plate- 1, 2).

Plate- 1 Nutmeg Tree

Plate- 2 Nutmeg

Oil Extraction: About 500gm of the leaves were cut into medium sized pieces and were hydro distilled using Clevenger type distillation apparatus[5]. Distillation process was done for 24 hours and the oil was extracted. The oil obtained was refrigerated at 5°C till further use.

Analysis of Essential Oil:

Mass spectrometry analysis was performed on a Shimadzu GC 17A QP 5000MS coupled with a mass detector, fitted non-polar DB-5(DiPhenyl Dimethyl Siloxane). Capillary column of length 25mx0.25mm Id. GC-MS operation conditions are initial temperature 60°C, programmed from 60°C - 300°C with the injection temperature at 260°C and detector temperature at 300°C. The injection volume was 0.1µl with helium gas as carrier at the flow rate of 0.6ml per minute. Relative retention times (RRts) of constituents were determined using C5 –C30 straight chain alkanes as standards. Individual constituents of the oil were identified by WILEY and NIST database matching by comparison of their RRts.

Antimicrobial Activity:

Oil extracts were subjected to the antimicrobial assay followed by Kirby Bauer method[6,7,8,9,10]. The filter paper discs of 5mm size were prepared and the extracted oil of *Myristica fragrans* was applied over the filter paper discs. The extract was evaporated after each addition and allowed to dry for 30 minutes. Six bacterial culture (Microbial Type Culture Collection- MTCC Collection) were maintained as pure cultures in Nutrient Agar slants with periodic sub-culturing was done every 4 – 5 days. Six fungal strains (MTCC Collection) were maintained as pure culture in Rose Bengal Agar slants and Potato Dextrose Agar slants with periodic sub-culturing was done every 7 -8 days. The plates were incubated at room temperature for three days. After three days inhibition zones including the diameter of the disc were measured using digital vernier caliper.

Histochemical Studies:

For Fluorescence microscopy and histochemical works, fresh plant materials are collected and serial hand sections were produced. To this thin hand sections specific fluorescent and histochemical reagents were added and observed under the fluorescent microscope. By the appearance of specific colour change of storage and cellular chemicals, the presence of various histochemicals will be identified. Fluorescent stains such as calcofluor, phluoroglucinol, can be used for the localization of separate histochemicals. In histochemical localization, localize specific cellular chemicals by using various histochemicals. Several histochemical dyes such as TBO, Sudan III, Oil red 'O' and I_2KI, can be used for the localization of phenolics, lipids, oil bodies and starch granules respectively.

Larvicide activity:

The mosquito larvae were collected in a sterile, disposable plastic container from a tank. The 5 larvae were transferred to a vial and 0.2ml of oil was added. The activity of

essential oil of *Myristica fragrans* against the larvae was noted. Another 5 larvae were transferred into a vial and 0.2ml of distilled water were added and kept as control.

Anticancer activity:

Viability Staining by Trtpan blue dye exclusion method:

Cytotoxic activity of oil extract were analysed by Trypan Blue dye exclusion method. MCF-7 breast cancer cell line and A375 cell line was used for the determination of cytotoxic activity. Cells were maintained in DMEM (Dulbeccos Modified Eagles Medium) supplemented with FBS (Foetal Bovine Serum) and penicillin/streptomycin-L-glutamine and cultured in a humified atmosphere of 5% CO2 and 95% air at 37°C in Thermo Hera Cell 150 incubator.

Cell lines in exponential growth phase were washed with phosphate buffer saline (PBS) solution and trypsinized and re-suspended in complete culture media. Cells were plated at 30,000 cells/well in 96 well plates and incubated for 24 hours during which a partial monolayer forms. After incubation the cells were exposed to various concentrations of the drugs, which is the plant oil extract (1000g/ml, 500g/ml, 250g/ml, 150g/ml, 125g/ml, 50g/ml and 25g/ml). The control well received only maintenance of medium. The plates were incubated at 37°C in a humidified incubator with 5% CO_2 for a period of 24 hours. Morphological changes of drug treated cells were examined using an inverted microscope and compared with the cells serving as control. At the end of 24 hours incubation, cytotoxic activity was determined.

Result:

In the present study, about 7ml of oil was extracted from the 500 g of *Myristica fragrans.* GC – MS Analysis indicated that the essential oil contained about 20 peaks. The composition of essential oil and its relative percentages are given in Table-1. Beta- pinene (22.69%), alpha-pinene (14.06%), alpha-thujen (13.93%) and p-menth 1-en-4-ol (10.53%) are as the major constituents and this leaf oil contains the minor constituents like camphene (3.92%), α-terpinene (3.42%), Limonene (9.56%) p-cymene (6.86%) Linalool (7.41%), beta myrcen (4.81%), alpha fellandrene (3.14%), 3-carene (3.54%), allyl catechol methylene (8.32%), myristicin (7.20%), elermicin (9.85%), beta ocimene (4.74%), gamma terpinene (4.75%), alpha terpinolene (4.38%), p-menth 2-en-1-ol (3.30%) and α-terpineol (6.00%). Chromatogram of essential oil extract was showed in fig- 1. The composition of essential oil and its relative percentages are given in Table-1.

Fig- 1: Chromatogram of *Myristica fragrans* Oil Extract

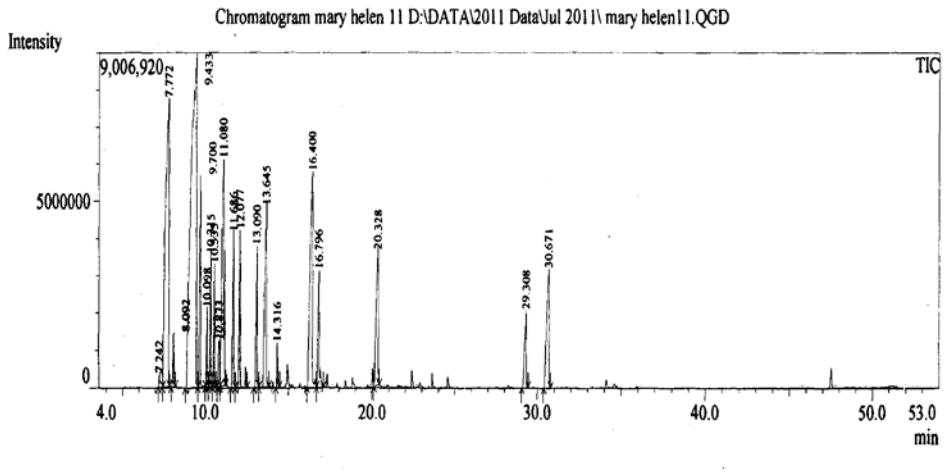


Table- 1: Percentage, Composition of Essential Oil from *Myristica fragrans:*

Number of Peaks	Retention Time (minutes)	Compounds	Abundance (%)
1	7.242	Alpha- Thujen	13.93
2	7.772	Alpha- Pinene	14.06
3	8.092	Camphene	3.92
4	9.433	Beta Pinene	22.69
5	9.700	Beta Myrcen	4.81
6	10.098	Alpha Fellandrene	3.14
7	10.315	3- Carene	3.54
8	10.535	Alpha- Terpinene	3.42
9	10.833	p- Cymene	6.86
10	11.080	Limonene	9.56
11	11.686	Beta- Ocimene	4.74
12	12.077	Gamma- Terpinene	4.75
13	13.090	Alpha- Terpinolene	4.38
14	13.645	Linalool	7.41
15	14.316	p- Menth 2-en-1-ol	3.30
16	16.400	p- Menth 1-en-4-ol	10.53
17	16.796	Alpha- Terpineol	6.00
18	20.328	Allylcatechol methylene ether	8.32

19	29.308	Myristicin	7.20
20	30.671	Elermicin	9.85

The antimicrobial activity of *Myristica fragrans* oil was tested against Six bacteria (*Enterococcus faecalis, Lactococcus plantarum Proteus vulgaris, Clostridium pertringens, Klebsiella Pneumoniae* and *Bacillus megaterium)* and six fungus (*Candida tropicalis, Candida albicans, Rhizomucor miehei, Candida glabrata, Aspergillus niger and Aspergillus fumigates).* Among the bacteria the maximum inhibitory effect was showed against *Enterococcus faecalis* (1.3cm), *Lactococcus plantarum* (0.9cm) and no inhibitory activity was observed against the bacteria *Clostridium pertringens, Klebsiella Pneumoniae* and *Bacillus megaterium*. In fungus *Candida tropicalis* showed high activity (1.3cm) and also *Candida albicans* (0.8cm), *Rhizomucor miehei* (0.6cm) and *Candida glabrata* (0.6cm). No inhibitory activity against *Aspergillus niger and Aspergillus fumigates.* The results were presented in Table 2 and 3.

Table- 2: Antimicrobial activity of essential oil against 6 bacterial strains by Kirby Bauer method:

Bacteria	**Zone of inhibition in 10µl sample(cm)**
Enterococcus faecalis	1.3
Lactococcus plantarum	0.9
Proteus vulgaris	0.6
Clostridium pertringens	0
Klebsiella Pneumoniae	0
Bacillus megaterium	0

Table-3: Antimicrobial activity of essential oil against 6 fungal strains by Kirby Bauer method

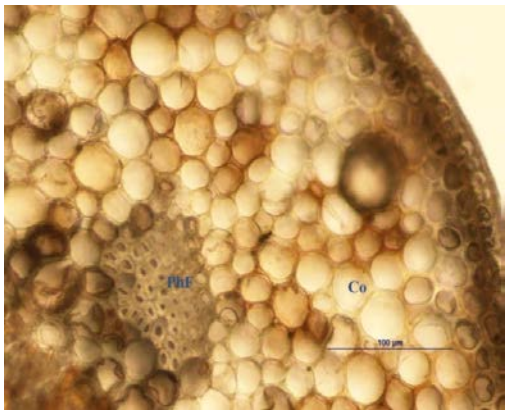
Fungi	**Zone of inhibition in 10µl sample(cm)**
Candida tropicalis	1.3
Candida albicans	0.8
Rhizomucor miehei	0.6
Candida glabrata	0.6
Aspergillus niger	Nil
Aspergillus fumigates	Nil

Histochemistry:

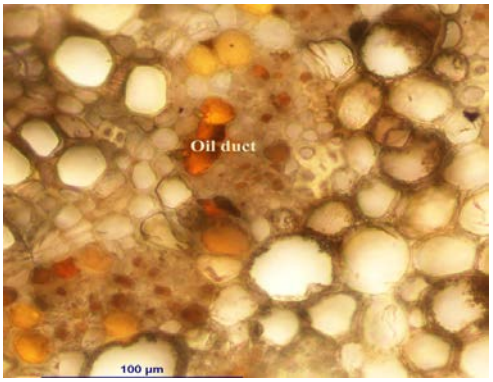
The fluorescent stains localize specific histochemicals. Localizations include cortex region, phloem fibres, oil ducts, phenol secreting cells, vascular cambial cells, phloem fibres, leaf midrib (Plate-3).

Plate-3 Histochemical Analysis of *Myristica fragrans*

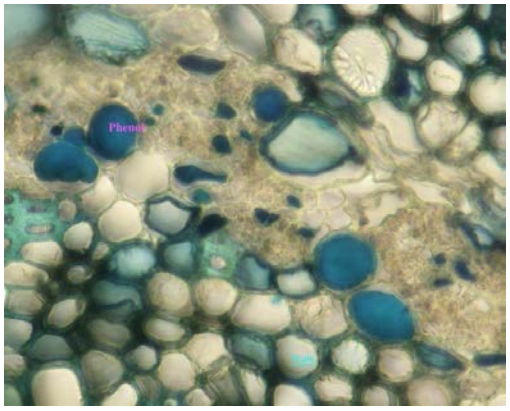
Cortex Region (Co) and Phloem Fibres (PhF)



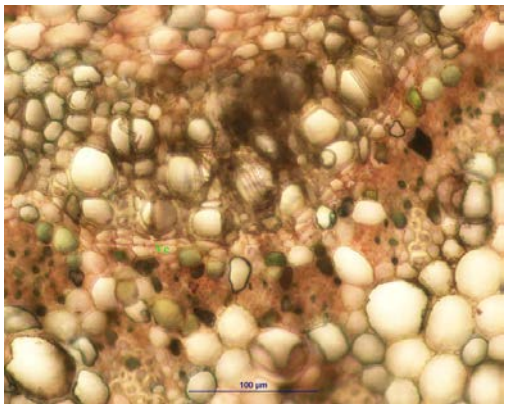
Oil Ducts



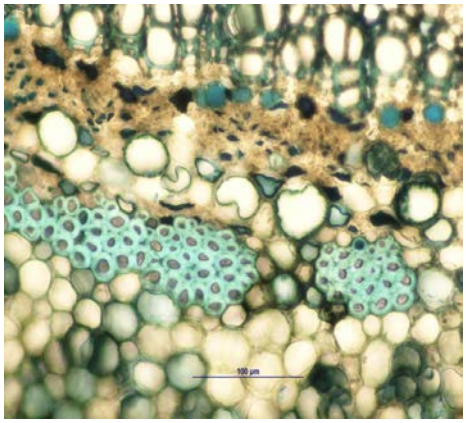
Phenol Secreting Cells



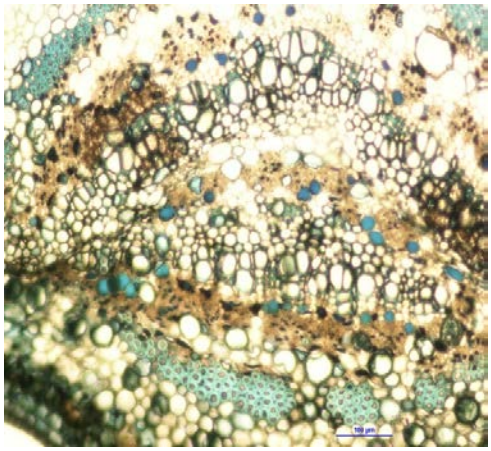
Vascular Cambial (VC)



Phloem Fibres



Leaf Midrib



Larvicide activity:

Mosquito larvae were susceptible to *Myristica fragrans* oil. The mortality of mosquito larvae was rapid. On addition of oil 100 percent mortality of mosquito larvae was observed within 50 seconds. Larvicidal activity of oil can be attributed to the presence of triterpenoides in oil. However the larvicidal activity cannot be attributed to any single constituent; rather it may be due to synergistic effect of various constituents (Table- 4).

Table- 4: Larvicidal Activity of *Myristica fragrans* Oil Extract:

Concentration of Oil Extract (ml)	Time (Seconds)	Larvae
0.2ml	50	5

Anticancer activity:

Viability Staining by Trypan blue dye exclusion method:

The cytotoxic effects of oil extract on MCF-7 and A375 cell lines by Trypan Blue dye exclusion method. The Trypan Blue dye exclusion method of MCF-7 cells after contact with 10µl, 25µl, 50µl, 75µl, 100µl extract showed 3%, 9%, 42%, 52% and 64% metabolic activity respectively. The result was showed in table –6 and plate- 3. The Trypan Blue dye exclusion method of A375 cells after contact with 10µl, 25µl, 50µl, 75µl, 100µl extract showed 9%, 18%, 30%, 37% and 49% metabolic activity respectively. The result was showed in table –7 and plate- 4.

Table-6: Cytotoxic Activity of Plant Oil Extract against MCF-7 Cell Line:

Concentration (µl)	Viable	Nonviable	Cytotoxic activity (%)
10	2011	59	3
25	1879	191	9
50	1200	870	42
75	985	1085	52
100	745	1325	64

Table- 7: Cytotoxic Activity of Plant Oil Extract against A375 Cell Line:

Concentration (µl)	Viable	Nonviable	Cytotoxic activity (%)
10	1952	207	9
25	1761	398	18

50	1505	654	30
75	1349	810	37
100	1105	1054	49

Plate- 3: Cytotoxic Effect of Oil Extract on MCF-7 Cell Line

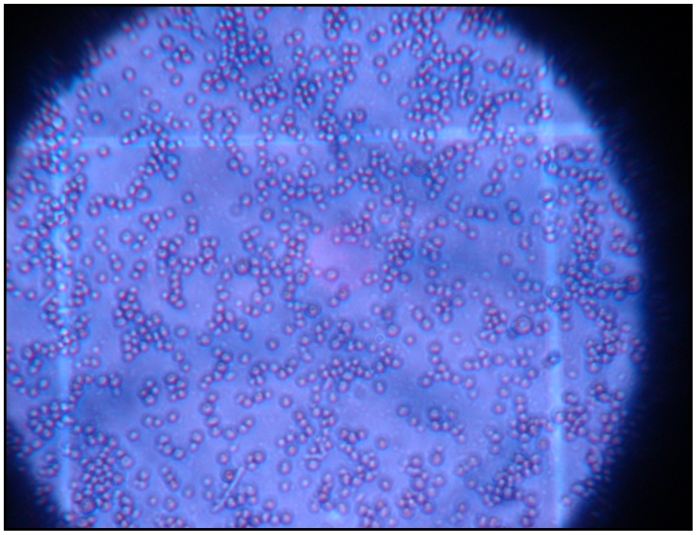
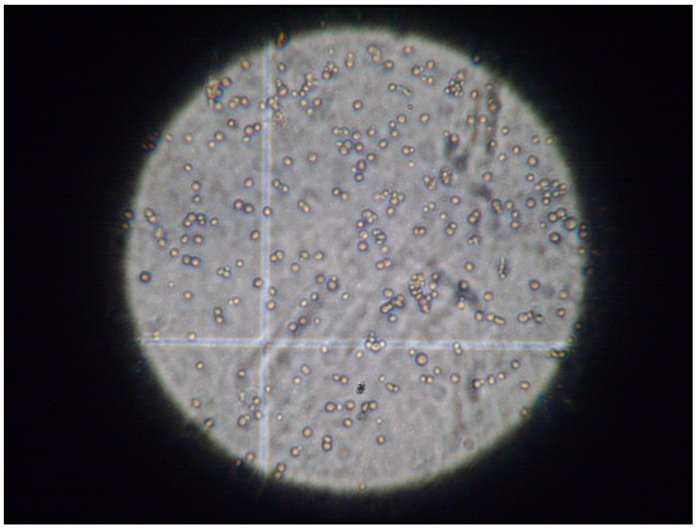


Plate- 4: Cytotoxic Effect of Oil Extract on A375 Cell Line



Discussion:

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. Oils such as sweet almond, carrot and mandarin were shown to possess little or no antimicrobial activity[6,9,11]. In the present study hydro distillation of fresh leaves, about 1.4% of pale yellow coloured, pleasant smelling oil was obtained. Similarly 1.2% of oil was obtained from this leaf of *Myristica fragrans*[12].

In GC/MS analysis, the essential oil of *Myristica fragrans* yielded 20 peaks. From this analysis the major compounds were identified as beta- pinene (22.69%), alpha-pinene (14.06%), alpha-thujen (13.93%) and p-menth 1-en-4-ol (10.53%). Similarly in *M. fragrans* leaf oil, identified the following major compounds: α-pinene, sabinene, 4-terpineol, limonene and β-pinene[13]. The oil showed significant activities against the food infectives and human pathogenic bacteria, *Proteus vulgaris* and the fungi *Candida tropicalis, Candida albicans.* The bacteria such as *Enterococcus faecalis* show high activity against essential oil *Enterococcus faecalis* will cause endocarditis, as well as bladder, prostate, and epididymal infections. So drugs can be made against these diseases using essential oil of *Myristica fragrans*[14], and antifungal activity against several pathogenic fungi including *Aspergillus flavus* and *Candida albicans*[15].

Histochemistry of leaves of *Myristica fragrans* showed localization of oil ducts, presence of phenol, vascular cambial cells and cortex. Essential oil of *Myristica fragrans* showed 100% larvicide activity. Similarly the seed at a dose of 1.0% of the diet produced activity on *Callosobruchus maculantus* larvae[16]. Essential oil showed significant cytotoxic activity against breast cancer cell line and epidermal skin cancer cell line. Water extract of dried kernel administered interaperitoneally to mice was effective on sarcoma 180[17].

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