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Extraction and identification of terpenes from *Myristica fragrans* (nutmeg) oil and comparing antibacterial property with the commercially available oil sample

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Abstract

Traditional herbal medicine is gaining demand as an alternative to chemical antibiotics as their side effects and drawbacks of comes into light. *Myristica fragrans* plant paves its way to become a boon to the medicinal community with its antibacterial and therapeutic applications. The phytochemicals present in nutmeg oil play a vital role in inhibiting the growth of undesirable bacterial strains. This study enable the concept of using phytochemicals to fight against pathogenic strains to progress by leaps and bounds. Furthermore, the study of the components present in nutmeg oil gives an idea as to which phytochemical, i.e. terpenoids is responsible for the antibacterial property of nutmeg oil by performing GC-MS for the oil. Therefore the study of the phytochemicals present in nutmeg and their effectiveness due to the presence of terpenoids, i.e. the requisite component responsible for the antibacterial activity of the oil can aid mankind in its approach towards effective and organic medicine. However, further studies need to be done for the identification of effective dosages for the production of antibiotic drugs.

Keywords: *Myristica fragrans*, Nutmeg Oil, Antibacterial property, *Bacillus* sp., *Sarcina* sp., *Lactobacillus* sp.

1. Introduction

Myristica fragrans Houtt, (nutmeg) is a ground spice belonging to the family of Myristicaceae and native to Moluccas, Spice Islands of Indonesia. This spice is infamous for enriching the flavors of the savory dishes of the Mughlai cuisine and many others and has been found to be a boon to the concept of traditional medicine. Composing majorly of alkyl benzene derivatives, this desiccated kernel of spacious ovoid-shaped seed, houses a plethora of phytochemicals such as alkaloids, flavonoids, steroids, tannins, resins, glycosides, phenols and most importantly terpenoids. These phytochemicals constitute for the very essence of the nutmeg plant that enlists a huge number of therapeutic uses.

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Research in the antimicrobial activity of both nutmeg and mace in various forms dates back to the 1990's. Study De *et al*., (1999) gives an insight into nutmeg's antimicrobial activity against *Bacillus subtilis* (ATCC 6633), *Escherichia coli* (ATCC 10536) and *Saccharomyces cerevisiae* (ATCC 9763).

Studies by Shinohara *et al*., (1999), Dorman & Deans (2000), Takikawa *et al*., (2002), O'Mahony *et al*., (2005), Mahady *et al*., (2005), Rani and Khullar (2004) have shown the inhibitory action of nutmeg extracts on a range of bacterial strains belonging to different genera including *Porphyromonas gingivalis, Acinetobacter calcoaceticus, Beneckea natriegens, Erwinia carotovora*, *Proteus vulgaris*, *Helicobacter pylori* and *Salmonella typhi*.

Gupta (2013) experimented the antimicrobial activity of various nutmeg extracts such as acetone, ethanol, methanol, butanol and water against pathogenic bacteria and fungi, specifically *Bacillus subtilis, Staphylococcus aureus, Pseudomonas putida, Pseudomonas aeruginosa,*

Aspergillus fumigatus, Aspergillus niger and *Aspergillus flavus*. The results of the study showed that all extracts of nutmeg shows bactericidal effect against the microbes. Acetone extract was found to show the greatest antimicrobial activity.

In the present study, effect of ethanolic extract of nutmeg oil of 4 different bacterial strains is studied using agar well diffusion method and comparison is made with the commercially available nutmeg oil.

2. Materials and methods

2.1 Sample Collection

Nutmeg seed (1000g) was collected from Sri Lakshmi Traders, Bendre Nagar of Bangalore in the month of September 2017 and was crushed into fine powder and stored in an air-tight container until use.

Commercially available nutmeg oil was purchased from Falcon Essential Oils, Banashankari 6th Stage, Bangalore 560060.

2.2 Extraction of Nutmeg Oil

Nutmeg oil was extracted by employing steam distillation method using Soxhlet apparatus. 50g of crushed nutmeg was taken in the distillation flask with 200ml of water and was heated at 80°C for 3 hours. Due to the heat, vaporization of volatile compounds occurred with steam and these vapors got condensed back to liquid in the coil and got collected as a top layer in the separator. The process was repeated for sufficient amount of oil (Al-Jumaily and Al-Amiry, 2012).

2.3 Evaluation of physical characteristics

Physical characters such as colour, odour, clarity, taste and solubility of the extracted nutmeg oil were evaluated.

2.4 Phytochemical Analysis

The nutmeg extract was screened for the presence of various phytochemical tests by using the following tests: Terpenoids and Tannins according to Aiyegoro and Okal (2010), Steroids according to Libermann-Buchard's Test, Resins according to Al-Balany (2004), Phenols according to Aljumaily (2004), Saponins according to Kokate (1999), Alkaloids according to Harbone, Glycosides according to Keller-Kiliani Testand Flavonoids according to Jaffer et al. (1983).

2.5 Gas Chromatography-Mass Spectrometry (GC-MS) Analysis

GC-MS Technique is employed to identify the components present in the oil. GC-MS of nutmeg oil was carried out by Bangalore Analytical Research Center, Peenay, Bangalore. The GC-MS of extracted nutmeg oil was obtained using Shimadzu GC-14A gas chromatograph with FID detector and SE-30 column (length and inner diameter). The operating conditions were 20mL/min constant flow of helium, hydrogen and air as carrier gases, oven temperature at 100°C for 2 minutes at the beginning and then increased at the rate of 10°C/min until 270°C. The injector temperature was kept at 250°C and detector temperature was kept at 300°C.

2.6 Media and Microorganisms

The microorganisms and media components employed in this research was procured from Microbiology Laboratory, Department of Microbiology, School of Sciences, Jain University. The cultures for which antibacterial test was carried out are Bacillus spp., *Bacillus clausii*, Sarcina spp and Lactobacillus spp.The strains were cultured on nutrient media and incubated at 37°C for 48 hours. The composition of nutrient media was beef extract 3.0g, peptone 5.0g, sodium chloride 8.0 g and agar 15.0 g. For antibacterial activity screening Mueller-Hinton agar was prepared using beef extract 3.0g, casein hydrolysate 17.5g, starch 1.5g and agar 17g.

2.7 Bacterial Sensitivity Test

Agar well diffusion method (Monica, 2003) was used to determine the antibacterial activity of the organisms. Mueller Hinton agar plates were incubated with 0.1 ml of 48 hour nutrient broth culture of each organism. Wells were made in the agar plates using a sterile cork borer and 100 ml of the extracted nutmeg oil were added to each culture plate. The plates were incubated overnight at 37°C and the diameters of zone of inhibition was measured and noted.

3. Results and comparative analysis

3.1 Physical characteristics evaluation of nutmeg oil

The extracted oil was a clear, brownish-yellow in colour with a strong, turpentine-like odour. The extracted nutmeg oil was found to be soluble in alcohol, ether and chloroform. It was insoluble in water.

3.2 Phytochemical Analysis

The ethanolic extract of nutmeg was found to contain terpenoids, tannins, resins, phenols, alkaloids, glycosides, flavonoids and steroids while saponins was absent as mentioned in Table I.

Table I: Phytochemical analysis results

Compound	Result
Terpenoids	Reddish brown layer formed on the inter-face
Tannins	Blue coloured solution formed
Resins	Turbidity observed
Phenols	Fresh reddish blue coloured solution formed
Saponins	No foam formation
Alkaloids	Yellow coloured precipitate formed
Glycosides	Brown ring formed between the layers
Flavonoids	Yellow coloured solution formed
Steroids	Solution colour changed from violet to bluish green

3.3 GC-MS Analysis

The GC-MS analysis of the extracted nutmeg oil, obtained from 3 hours water distillation showed the presence of 36 peaks representing 99.21% of the total extract. The concentration and retention times of each of these components were identified through the analysis. The retention times were in the range 5.10 to 33.97. The identification of suspected peaks is mentioned in Table II.

The percentage area corresponds to the concentration of component present. The major components of extracted nutmeg oil as concluded from the peaks included; a-pinene, β-pinene, sabinene, elemicine, myristicine, 4-terpineol, terpinolene, safrene, g-terpinene and methyleugenol. The most dominant component was found to be sabinene at 27.7% (Figure I).

3.4 Antibacterial Activity Analysis

The sensitivity of both extracted nutmeg oil and commercially nutmeg oil against four bacterial species was tested by measuring the diameter of zone of inhibition (Figure II (A-D)). It was observed that the extracts of nutmeg used in the present study possess considerable antimicrobial activity against the tested microorganism. The results are as mentioned in below Table III.

Both extracted nutmeg essential oil and the commercially available oil had the most profound inhibitory action against *Bacillus clausii* while the least activity was

Table II: GC-MS analysis

Sl. No.	Apex RT	Area	%Area	Identification
1	9.25	138654056.2	0.97	α-Thujene
2	9.47	925210358.7	6.45	α-Pinene
3	10.73	3974245584	27.7	Sabinene
4	10.88	1009706121	7.04	(-)-β-Pinene
5	11.25	222259672.1	1.55	(-)-β-Pinene
6	11.78	52603482.98	0.37	α-Fellandrene
7	11.85	153815217.4	1.07	delta-3-Carene
8	12.12	303523559.8	2.12	Terpinolene
9	12.37	183811797.6	1.28	p-Cimene
10	12.51	212944111.3	1.48	D-sylvestrene
11	12.57	106264287.3	0.74	Phellandrene, β
12	12.64	24886338.01	0.17	Cineole
13	13.43	550604611.7	3.84	γ-Terpinen
14	13.85	25641819.34	0.18	cis-2-Menthenol
15	14.28	278650995.3	1.94	Terpinolene
16	14.44	9262907.312	0.06	β-Linalool
17	14.75	37622968.36	0.26	trans-β-Terpineol
18	14.82	10465571.44	0.07	cis-β-Terpineol
19	15.53	71173673.05	0.5	trans-2-Menthenol
20	16.08	54208923.07	0.38	cis-2-Menthenol
21	17.22	1453485900	10.13	(-)-4-Terpineol
22	17.65	128467645.6	0.9	α-Terpinol
23	20.35	575781270.3	4.01	Safrene
24	21.9	160118165.7	1.12	δ-EIemene
25	22.05	30690219.28	0.21	trans-Isoeugenol
26	22.7	178778862.2	1.25	Copaene
27	23.29	348852571.5	2.43	Methyleugenol
28	23.88	106604981.7	0.74	Caryophyllene
29	24.17	37538537.63	0.26	cis-α-Bergamotene
30	24.8	13713374.25	0.1	cis-α-Bisabolene
31	25.44	71162055.28	0.5	β-Cubebene
32	25.72	52641042.37	0.37	Methylisoeugenol
33	26.06	19776096.13	0.14	α-Himachalene
34	26.37	1293602260	9.02	Myristicine
35	26.97	1373483729	9.57	Elemicin
36	29.32	42229921.06	0.29	Isoelemicin

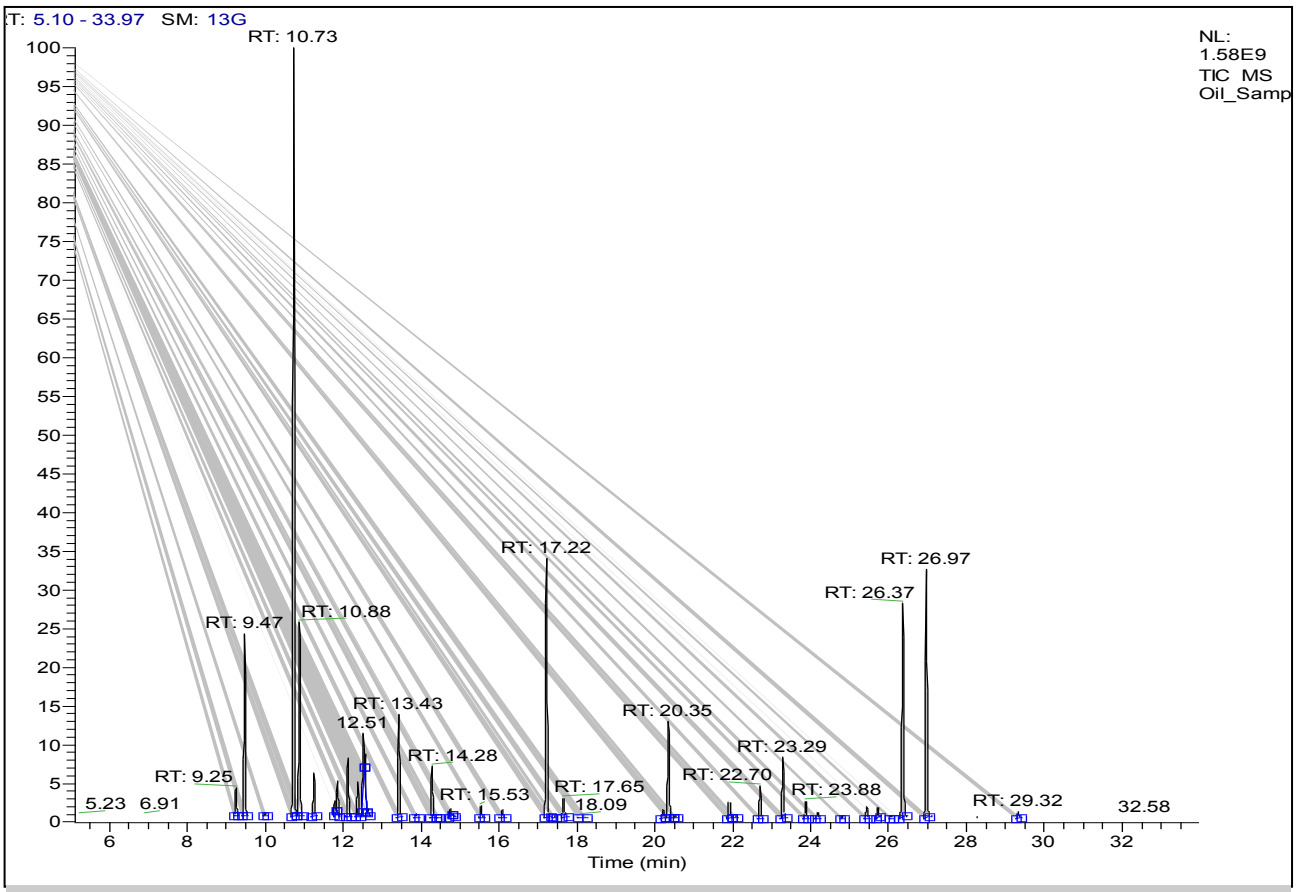

Figure I: Components Identified by GC-MS Analysis

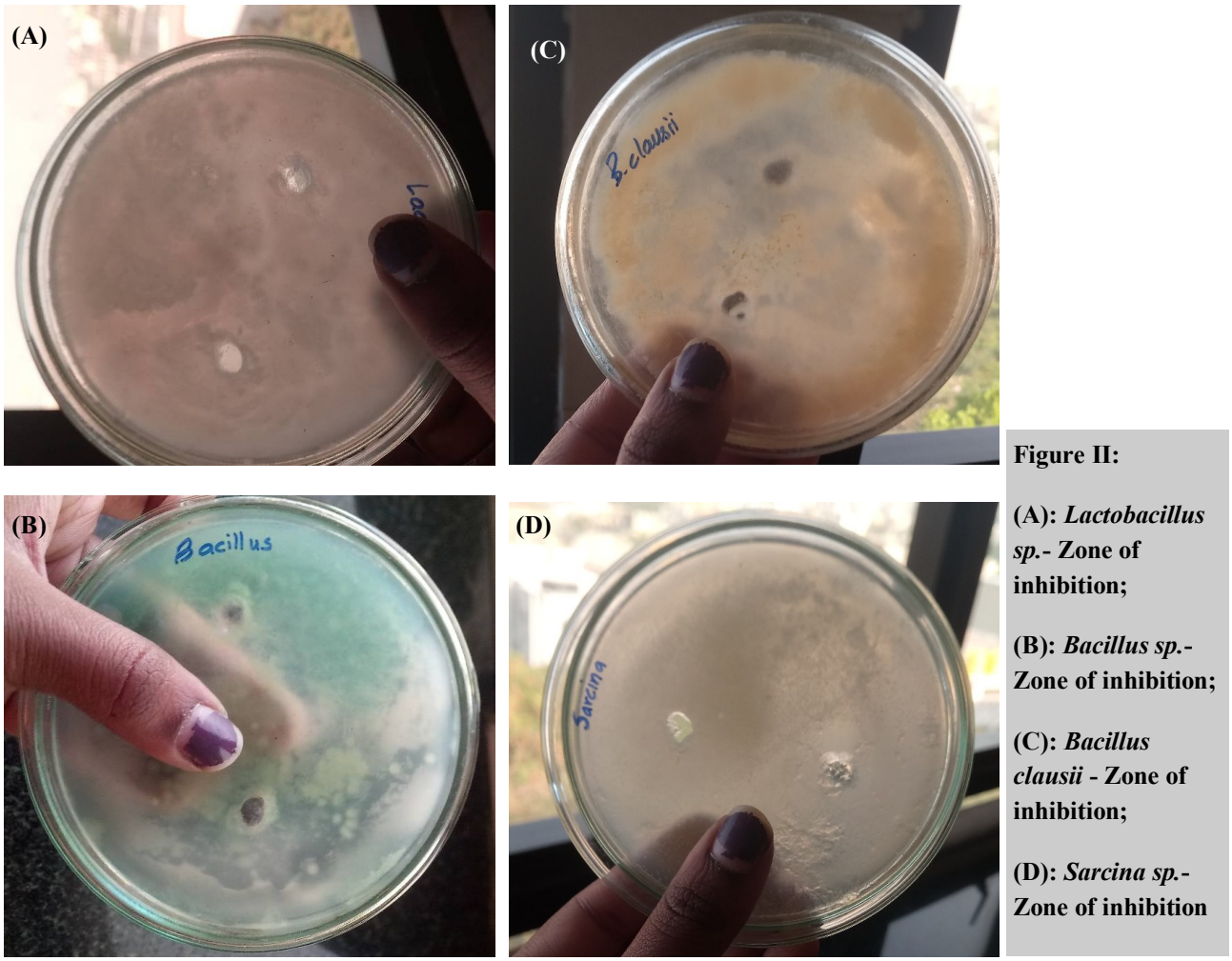

Figure II:

(A): *Lactobacillus sp.*- Zone of inhibition;

(B): *Bacillus sp.*- Zone of inhibition;

(C): *Bacillus clausii* - Zone of inhibition;

(D): *Sarcina sp.*- Zone of inhibition

Table III: Antibacterial property analysis

Bacterial Species	Diameter of Zone of Inhibition (cm)			
	Bacillus spp.	*Bacillus clausii*	*Lactobacillus spp.*	*Sarcina spp.*
Extracted Nutmeg Essential Oil	2.3	2.5	1.8	2.1
Commercially Available Nutmeg Essential Oil	2.3	2.5	2.0	2.1

observed against *Lactobacillus spp.,* with the extracted nutmeg essential oil. It was observed that the nutmeg essential oils used in the present study possess considerable antimicrobial activity against the tested microorganism.

3.5 Comparative Analysis

Comparing the results of both extracted nutmeg essential oil and the commercially available nutmeg essential oil showed little to no difference in the zone of inhibitions for all the bacterial strains tested. Only species which showed difference in zone of inhibition was Lactobacillus species and it was a difference of 2cm. This indicates both essential oils were pure with no added ingredients and is efficient.

4. Discussion

This study explores the correlation between the antibacterial properties of nutmeg oil and the various chemical compounds present in the oil. All the strains for which antibacterial activity were gram positive bacterial strains. The difference in zone of inhibition may be due to the difference in physiological and biochemical characteristics between the strains. This is indicated by the small difference in inhibitory action of nutmeg towards *Bacillus clausii* and *Bacillus spp.,* as they belong to the same genera.

Many components which was identified by GC-MS analysis are known for their medicinal and therapeutic properties. From the components found to occur in highest concentration in the oil, most belong to the family of terpenes, specifically to the class of monoterpenes. These compounds are a-pinene (6.45%), β -pinene (8.59%), sabinene (27.7%), terpinolene (2.12%), g- terpinene (3.84) and 4-terpineol (10.13%). The other three components elemicine (9.57%), myristicine (9.02%) and methyleugenol (2.43%) which were present in highest amount belonged to the class of phenylpropanes. Almost all of these components have been pronounced by various literature for their antimicrobial and therapeutic properties.

According to Appian Subramonium (2014), sabinene shows a great antibacterial effect against *Salmonella typhi*. Also it has been proved that it exhibit antibacterial property against gram positive bacterial strains at different levels.

Study by Sean Tighe, Ying-Ying Goa and Scheffer C. G. Tseng (2013) has shown that 4-terpineol which is the most active and predominant ingredient present in tea tree oil contributes to its parasitism against Demodexmites. In addition to this, 4- terpineol has been tested for the treatment of bovine mastitis and thrush in horses due to its antimicrobial properties.

Yuangang Zu (2012) showed in his study the antibacterial property of β-pinene found in nutmeg extract against gram positive bacteria. Takikawa et al., have reported that *Escherichia coli O157* is highly sensitive to β-pinene. The study also concluded that β-pinene present in *Rosmarinus officinalis L.* essential oil exhibit antibacterial activity to a lesser extend as compared toα-pinene. In addition to this, studies have also revealed that α-pineneshows bactericidal activity against *Staphylococcus aureus*.

According to a study carried out by Seyedeh Mahsan Hoseini-Alfatemi and Marcello Iriti (2015) the main active ingredient of Satureja intermedia C.A.Mey Essential Oil which shows the antibacterial activity against *Streptococcus salivarius, Enterococcus faecalis, Streptococcus mutants, Staphylococcus aureus* is g-terpinene.

The antibacterial property of terpinolene is observed in a study done by Fereshteh Eftekhar (2005). His study revealed that*Diplotaenia damavandica* essential oil which has terpinolene as one of its major components exhibit antibacterial activity against *Bacillus subtilis, Enterococcus faecalis, Escherichia coli, Staphylococcus epidermidis, Staphylococcus aureus* and *Pseudomonas aeruginosa*.

Evidence was provided by Rossi (2007) that elemicine, which is one of the two key ingredients in *Daucus coarota L.*essential oil exhibits bactericidal activity against *Campylobacter jejuni.* Antibacterial activity of myristicine was shown by Narasimhan and Dhake (2006) who reported myristicine exhibited good antibacterial activity against both gram positive and gram negative bacteria.

Evidence in literature proves the medicinal and therapeutic properties of the major components found to be present in the extracted nutmeg oil. The components identified in GC-MS analysis can be classified based on the family of compounds they belong to. About 72.19 %

of the extracted essential oil comprises of terpenes while about 27.02 % belong to the family of phenylpropanes. Due to the high percentage of terpenes, the bactericidal activity of the extracted essential oil can be correlated with these components. The remaining components contribution would be to a minimum.

Further purification of these components is required to test their minimum inhibitory concentration which can help in the standardization of an effective dosage and its commercial production for medicinal products such as tablets and syrups.

5. Conclusion

The research findings conclude that essential oil extracted from nutmeg contains phytochemical compounds such as terpenoids which contribute to its antibacterial property. Additionally the nutmeg oil available commercially which has no added ingredients is as efficient as pure nutmeg oil extracted in exhibiting medicinal properties. The highest inhibitory action of nutmeg oil was observed against *Bacillus clausii*. These results therefore supports the traditional usage of essential oils for medicinal purposes. However further studies are necessary to examine the underlying mechanisms of the phytochemical constituents responsible for these pharmacological activities.

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Conflict of interest

Authors declare none

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