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STUDY OF ANTIBACTERIAL EFFECT OF *MYRISTICA FRAGRANS* OIL ON PLANKTONIC AND SESSILE ESBL AND MBL PRODUCERS

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ABSTRACT

The major threat of antibiotic resistance has left the clinical society vulnerable to common infectious diseases with the emergence of Extended-Spectrum β-lactamase (ESBL) and Metallo β-lactamase (MBL) producing pathogens. This impuissant situation demands alternative approaches and strategies to manage complicated infections. One such option is the use of primitive herbal medicinal sources that effectively comply with our current needs for side-effect free supplements. The present study investigated the antibacterial activity of Myristica fragrans (nutmeg) oil on 25 representative isolates of ESBL and MBL producing pathogens respectively. Owing to the importance of biofilms in the degree of pathogenicity observed in bacterial infections, the activity was studied on both sessile and planktonic forms of test isolates. The biofilm formation was confirmed by using Congo red and tube method. The antibacterial activity of M. fragrans was clearly evident with the zones of inhibition (15-25mm) showed by agar diffusion method, and their observed Minimum Bactericidal Concentration (0.3% v/v). Moreover, the sub-inhibitory concentration of M. fragrans successfully inhibited the biofilm formation in test isolates. The GC-MS analysis showed the presence of terpenes and other aromatic compounds which are known for its antimicrobial property. These results collectively indicate the possible use of M. fragrans oil to treat infectious diseases caused by multiple drug-resistant pathogens.

KEY WORDS

Myristica fragrans, Nutmeg oil, ESBL, MBL, Antibacterial

INTRODUCTION

Over the years, the increasing prevalence of antibiotic-resistant pathogens has significantly limited the effectiveness of current drugs resulting in treatment failure. The current scenario coupled with the common infections like Urinary Tract Infections (UTIs) has intensified the concerns related to the ease of spread of antibiotic resistance in hospitals as well as community settings [1]. The succoring advent of Extended-Spectrum β-Lactamase (ESBL) and Metallo-β-lactamase (MBL) producers, especially among uro-pathogens, has further sealed the perpetual fate of clinicians to fight the challenges of antibiotic resistance [2]

ESBLs and MBLs are enzymes produced by pathogenic bacteria that are capable of hydrolyzing 3rd generation cephalosporins viz., cefuroxime, cefotaxime, ceftazidime and even carbapenem antibiotics [3, 4]. They also show a high degree of resistance to other groups of antibiotics [5]. In addition, the pathogens can also surpass the mechanical interference, host defenses as well as other treatment methods by forming sticky agglomerations known as “biofilms” [6]. The biofilm forming uro-pathogens are clinically important because they are involved in the colonization of the bladder via urinary catheters and also in the formation of intracellular bacterial communities [7].

Bacterial resistance owing to genetic mutation or exchange of plasmid(s) becomes easier in sessile

environment observed in biofilms. The resilience of biofilm-forming pathogens towards adverse survival conditions arises from factors like impaired diffusion of toxins and alterations in enzyme activity [8]. Evidently, there are reports suggesting the possibility of accumulation of antibiotic-inactivating enzymes such as β-lactamases within the glycocalyx which produce concentration gradients and hence protect the underlying cells [9]. Planktonic cells, on the other hand, may not be able to withstand the host immune responses and resist these higher antibiotic concentrations in spite of their acquired resistance [10]. The above-mentioned properties of antibiotic resistance along with bacterial strategies of biofilm formation have succeeded in persuading the clinical society to develop new antimicrobial compounds or modify the existing ones in order to improve antimicrobial therapies [11]. This, in turn, has resulted in a resurgence of phytotherapeutic approaches to challenge the existing views on allopathic pharmacopeia. Traditionally, the extracts and essential oils obtained from medicinal plants have been used extensively for the treatment of respiratory tract infections [12], sinusitis [13], inflammation of the trachea [14] and even asthma [15]. More recently several plant extracts have shown promising results for the treatment of chronic diseases like cancer [16] and multi-drug resistant infections [17]. Today, there is a much-developed interest in the essential oils obtained from different parts (bark, stem, leaves or seed) of several plants to effectively eliminate oxidants from the body and enhance the immunity. It is also used extensively in aromatherapy to fight stress-related disorders [18]. The secondary metabolites in the essential oils may be responsible for its range of observed activities [18, 19].

In spite of the developed interests and a decent amount of research undertaken in the field of primitive medicines, the information available to scientists is not sufficient for preparing effective formulations [20]. One of the major hurdles in this path is the variations observed in the quality and composition of essential oils. These variations may arise due to avoidable as well as unavoidable factors like extraction protocols, plant age, season, soil condition, cultivation techniques and cell cycle to name a few [21, 22]. However, in spite of these standardization issues, the market for plant medicinal products stands strong. Moreover, it is expected that plant compounds with diverse activity will be available for effective use against drug-resistant microbial pathogens very soon [20].

Considering the current clinical adversity of increasing antibiotic resistance among pathogens, the objective of our study was to investigate the efficacy of *Myristica fragrans* (nutmeg) essential oil against sessile and planktonic forms of ESBL and MBL producers.

MATERIALS AND METHODS

Test organisms

Gram-negative uro-pathogens isolated and characterized for ESBL and MBL production in a previous study was used as test organisms [23, 24]. Twenty-five representative ESBL and MBL producers including *Klebsiella pneumoniae*, *Escherichia coli, Pseudomonas aeruginosa, Proteus mirabilis* and *Citrobacter diversus* were used in the current study. These isolates were maintained on Luria-Bertani (LB) agar slants supplemented with 100µg/ml of ampicillin and stored at refrigerated conditions.

Plant material

Readily available oil extracted from *Myristica fragrans* (nutmeg) was purchased from a local ayurvedic pharmacy shop, Mumbai.

Sterility testing of *M. fragrans* oil

The sterility of the *M. fragrans* oil was confirmed by checking for bacterial or fungal growth after spot inoculating it on a sterile Nutrient Agar (NA) and Sabouraud's Agar (SAB) plate respectively [25]. The NA plates were incubated at 37°C and SAB plates at Room temperature (RT) for an extended duration of 7 days to confirm the absence of contaminants.

Detection of Biofilm formation by Congo red Agar method (CRA)

Brain heart infusion broth (BHI) supplemented with 5% sucrose and 0.8% Congo red was used for qualitative assessment of biofilm formation as described by Freeman et al. 1989 [26]. Congo red was prepared as a concentrated aqueous solution and autoclaved at 121°C for 15min, separately from other medium constituents and then added when the agar was cooled to 45°C. These plates were streaked with test isolates and incubated aerobically for 24h at 37°C. Biofilm forming cultures showed black colored colonies with dry crystalline centers and biofilm negative cultures were identified as pink or colorless.

Detection of Biofilm formation by Tube method (TM)

A qualitative assessment of biofilm formation was determined as previously described by Christensen et al. 1980.Sterile Trypticase Soy Broth supplemented with 2% glucose (10mL) was inoculated with a loop-full of overnight grown test pathogens and incubated for 24h at 37°C. The tubes were decanted and washed with Phosphate Buffered Saline (PBS pH 7.2), dried, and stained with crystal violet (0.1%). Excess stain was removed, and tubes were washed with distilled water. They were dried in an inverted position at RT and observed for biofilm formation.

Biofilm formation was considered positive when a visible film lined the wall and bottom of the tube. Ring formation at the liquid interface was not indicative of biofilm formation. Tubes were examined, and the amount of biofilm formation was scored as 0-absent, 1-moderate or 2-strong. All experiments were performed in triplicates [27].

Evaluation of the antibacterial efficiency of *M. fragrans* oil by well diffusion method

The *M. fragrans* oil was dissolved in ethanol and checked for its antibacterial activity against test pathogens with the help of agar well diffusion method. For this purpose, sterile 20ml molten NA butts were cooled to around 40°C, seeded with 0.4ml of 24h old test cultures (Macfarland tube 1) and poured into sterile petri-plates. Wells were punched in each plate after solidification of the medium using a sterile cork-borer (8mm diameter), and 50µl of the *M. fragrans* oil was added to it. The plates were incubated at 37°C for 24h to observe the zones of inhibition. Control wells were also set up using 50µl of ethanol.

Evaluation of MBC of *M. fragrans* oil by Agar dilution method

The agar dilution method was used to determine the MBC of *M. fragrans* oil. Different concentrations of oil were dissolved in ethanol (0.1-0.5% with an interval of 0.1%) were supplemented into molten NA butts cooled to around 40°C. After solidification of the medium, 5µl of test isolates were spot inoculated on the plates and incubated at 37°C for 24h. MBC was defined as the lowest concentration of *M. fragrans* oil that completely inhibited the growth of test cultures [28].

Inhibition of biofilm formation

Biofilm inhibition by *M. fragrans* oil was assessed by crystal violet staining method. The test isolates were allowed to grow overnight in nutrient broth supplemented with 100µl ampicillin. They were then transferred to 5ml sterile trypticase soy broth (TSB) containing a sub-MBC concentration of *M. fragrans* oil and the density was adjusted to 10^8 cfu/ml using Mac Farland's tube-1 standard opacity tubes. After 24h incubation at 37°C, the liquid culture was decanted and washed twice with distilled water. The tubes were then stained with 0.1% crystal violet for 5min. Excess stain was decanted, and the tubes were air dried. The dye retained in each test tube was recovered using 5ml ethanol and quantified using a spectrophotometer at a wavelength of 595nm. A control was also set up with 5ml sterile TSB without *M. fragrans* oil.

Bioautography of nutmeg oil

The bioactive components of *M. fragrans* oil were separated using paper chromatography. The different solvent systems used for this purpose were

1. Toluene: Methanol: Acetone: Acetic Acid (12:6:1:1)
2. Toluene: Methanol: Ethyl acetate (6:12:2)
3. Butanol: Acetone: distilled water (2:2:1)

The experiment was performed in triplicates. Out of the three sets used in our study, one set was used as a control and other 2 sets were developed using 1% iodine solution. After separation of the bioactive components on chromatography paper, they were placed on sterile petri-plates and overlaid with NA medium containing 0.03% Tetrazolium tri-chloride (TTC) and 0.1ml test culture. These plates were incubated at 37°C for 24h and observed for zones of inhibition by active bio-components in *M. fragrans* oil. Inhibition was confirmed as colorless zones against pink color matt growth of test isolates developed due to the presence of TTC [29].

Gas Chromatography-Mass Spectrophotometry analysis

The bioactive component from the *M. fragrans* oil was analyzed with the help of Gas Chromatography-Mass Spectrophotometry (GCMS). The GC-system was equipped with a capillary column of dimensions 30m X 0.25mm X 0.25µm. The program used for GC oven temperature was 5 min isothermal at 300ºC, followed by 90º-280ºC at a rate of 6ºC/min, then held at 280ºC for 5 min. The injection port temperature was 240ºC.

Along with that a Joel, AccuTOF GCV MS system, with a time of flight analyzer, was used [17]. The entire analysis was carried out at IIT Bombay, Mumbai 400076.

RESULTS AND DISCUSSION

Sterility testing of solvent extracts

The *M. fragrans* oil showed absence of bacterial and fungal contaminants even after 7 days of incubation. The extended incubation time confirmed the absence of slow-growing contaminants and stressed cells that may have survived the processes involved in the packaging of oil.

Detection of Biofilm formation by test organisms

Tables 1 and 2 represent the biofilm forming ability of the ESBL and MBL producers as observed by different methods used in the study. It was classified as grade 0, grade 1 or grade 2 biofilm producers depending on the extent of biofilm production observed. Consequently, high variability was observed, and classification of grades was difficult by tube method. Hence, Congo red agar method was carried out to confirm the biofilm formation by test isolates. Similar to our findings, it has been reported previously that the tube test cannot be recommended as a general screening test to identify biofilm production in test isolates [27].

Table 1: Biofilm production by ESBL producers

Sr. No.	Test cultures	Congo red Agar method	Tube method
1	*C. diversus* 116	Grade I	Grade I
2	*K. pneumoniae* 507	Grade II	Grade I
3	*E. coli* 223	Grade O	Grade O
4	*E. coli* 310	Grade O	Grade O
5	*E. coli* 63	Grade I	Grade I
6	*C. diversus* 500	Grade I	Grade O
7	*E. coli* 313	Grade O	Grade I
8	*P. vulgaris* 130	Grade I	Grade I
9	*E. coli* 74a	Grade II	Grade II
10	*C. diversus* 497	Grade II	Grade I
11	*K. pneumoniae* 645	Grade II	Grade II
12	*K. pneumoniae* 306	Grade O	Grade I
13	*E. coli* 96	Grade I	Grade I
14	*E. coli* 70	Grade I	Grade I
15	*E. coli* 289	Grade I	Grade I
16	*K. pneumoniae* 68	Grade I	Grade I
17	*E. coli* 39	Grade I	Grade I
18	*E. coli* 250	Grade II	Grade II
19	*E. coli* 267	Grade II	Grade II
20	*C. diversus* 28	Grade II	Grade II
21	*E. aerogenes* 60	Grade II	Grade II
22	*P. mirabilis* 9b	Grade I	Grade I
23	*E. coli* 115	Grade II	Grade II
24	*C. diversus* 30	Grade I	Grade I
25	*E. coli* 526	Grade II	Grade II

Table 2: Biofilm production by MBL producers

Sr. No.	Test cultures	Congo red Agar method	Tube method
1	*E. coli* 617	Grade II	Grade II
2	*P. aeruginosa* 85	Grade II	Grade II
3	*E. coli* 86	Grade II	Grade II
4	*E. coli* 469	Grade I	Grade I
5	*E. coli* 519	Grade I	Grade O
6	*E. coli* 459	Grade I	Grade I
7	*K. pneumoniae* 95	Grade I	Grade I
8	*E. coli* 479	Grade I	Grade O
9	*E. coli* 624	Grade I	Grade I
10	*P. mirabilis* 605	Grade I	Grade O
11	*E. coli* 467	Grade I	Grade I
12	*E. coli* 75	Grade O	Grade O
13	*E. coli* 493	Grade II	Grade II
14	*P. mirabilis* 607	Grade I	Grade O
15	*E. coli* 461	Grade I	Grade I
16	*E. coli* 526	Grade II	Grade II
17	*E. coli* 463	Grade O	Grade I
18	*E. coli* 80	Grade O	Grade I
19	*E. coli* 520	Grade I	Grade I
20	*E. coli* 534	Grade I	Grade I
21	*E. coli* 220	Grade II	Grade II
22	*E. coli* 234	Grade I	Grade I
23	*P. mirabilis* 608	Grade I	Grade I
24	*K. pneumoniae* 614	Grade II	Grade I
25	*P. mirabilis* 604	Grade O	Grade O

In our study, most of the organisms showed moderate to high production of biofilms. Out of the tested ESBL producers, 10 isolates were classified as grade 2 (high biofilm producer), 11 isolates were grade 1 (moderate biofilm producers), while 4 isolates showed no biofilm production. Among the MBL producers, 7 isolates were grade 2, 14 isolates were grade 1, and 4 isolates did not produce biofilms. All the isolates used in the study showed a high degree of resistance towards β-lactams, Fluoroquinolones, and cephalosporin antibiotics. Moreover, all the MBL producers and few ESBL producers also showed resistance to carbapenem antibiotics which are considered to be the last resort drugs [23, 24]. A similar study demonstrated 53% of total isolated strains of uropathogenic *E. coli* to be biofilm producers [30]. In addition, a significant correlation between biofilm production and resistance to multiple antibiotics such as ampicillin, cotrimoxazole, nalidixic acid and norfloxacin has been reported by other researchers [7, 30, 31].

Evaluation of the antibacterial efficiency of *M. fragrans* oil by well diffusion method

There was no significant difference in the observed zones of inhibition against ESBL and MBL producers and it was found to be in the range of 15-25mm (Table 3).

Table 1: Antibacterial activity of *M. fragrans* oil observed against ESBL and MBL producers

Test cultures	Zone of inhibition in mm	MBC (% v/v)
ESBL producers		
E. coli	15.33 - 20.67	0.03
C. diversus	20.42 - 22.67	0.03
K. pneumoniae	15.67 - 20.75	0.03
P. vulgaris	19.53 - 20.33	0.03
P. mirabilis	18.68 - 19.84	0.03
P. aeruginosa	20.56 – 22.67	0.03
MBL producers		
E .coli	15.33 – 25.33	0.03
K. pneumoniae	17.47 - 22.86	0.03
P. mirabilis	18.67 - 22.33	0.03
P. aeruginosa	20.67 - 21.67	0.03

Table 4: Inhibition of biofilm using crystal violet assay

Test cultures	O.D(λ_{max}=595nm)	
	Control	Test
ESBL producers		
K. pneumoniae 507	0.206	0.1435
E. coli 74a	0.1856	0.0638
C. diversus 497	0.2518	0.0906
K. pneumoniae 645	0.1590	0.0589
E. coli 250	0.3277	0.1232
E. coli 267	0.5016	0.2724
C. diversus 28	0.4029	0.1287
E. aerogenes 60	0.2322	0.1390
E. coli 115	0.6476	0.1369
E. coli 526	0.6802	0.1454
MBL producers		
E. coli 617	0.3476	0.1386
P. aeruginosa 85	0.5645	0.2736
E. coli 86	0.6983	0.1768
E. coli 493	0.6476	0.5369
E. coli 526	0.6802	0.6454
E. coli 220	0.4475	0.4336
K. pneumoniae 614	0.5948	0.5827

The antibacterial activity of *M. fragrans* oil is very well known. Similar to our observations, a recent study also showed inhibition zones of gram-negative (*E. coli*-MTCC 443, *P. vulgaris*-MTCC 1771, and *K. pneumoniae*-MTCC number 7028) as well as gram-positive (*S. aureus*-MTCC 3381, *B. subtilis*-MTCC 10619, and *B. megaterium*-MTCC 2412) test organisms in the range of 14-28mm [32]. Another study reported zones of inhibition of test isolates in the range of 07-13mm by hot and cold extracts of *M. fragrans* [33].

Although, a recent study published as conference proceedings reported the activity of *M. fragrans* against pathogenic bacteria [34]; to our knowledge, there are no studies available online that represents the efficacy of *M. fragrans* oil against ESBL and MBL producers exclusively.

Evaluation of MBC of *M. fragrans* oil by Agar dilution method

The MBC of *M. fragrans* oil against ESBL and MBL producers was found to be 0.03%v/v for all test isolates (Table 3). Another study reported the MBC of methanolic extract of *M. fragrans* against 29 antibiotic-resistant isolates to be in the range of 64-1024µg/mL [35]. The crude extracts of other plants like *Parkia javanica* have shown an MBC in the range of 5-20mg/mL [36] and that of *Dracontomelon dao* to be in the range of 3-4mg [37].

The standardization of plant extracts and essential oils is an impeding factor in the development of alternative

treatment plans. Comparative studies reported above show an extensive variation in the observed zones of inhibition and MBC values, clearly reflecting the problems associated with plant standardization protocols. These variations may be attributed to several underlying factors like the plant's biochemical composition. It is a well-known fact that the same plant collected from different areas may give rise to variations in the study. Other factors that may contribute to such variations may include the type of solvent used for extraction of bioactive components, extraction protocol, type of media and inoculum size among others [21, 22].

Inhibition of biofilm formation

The ability of sub-inhibitory concentration of *M. fragrans* oil to prevent biofilm formation was assessed using the crystal violet assay for grade 2 biofilm producers. Our study showed a clear reduction in the biofilm forming ability of the ESBL producers. However, 4 MBL producing isolates were not affected in presence of a sub-inhibitory concentration of *M. fragrans* oil (Table 4). A similar study carried out using aqueous and acetone extracts of *Coccinia grandis* showed a varied response in inhibition of biofilms formed by uropathogenic *E.coli*. However, the ethanolic extracts exhibited a better anti-biofilm activity [38]. In another study, the ethanolic extract of *Zingiber officinale* demonstrated moderate anti-biofilm activity against gram-negative test isolates, with the best activity being observed on *P. mirabilis*. They calculated the percentage reduction in the biofilm formation of *P. aeruginosa* ATCC 27853 to be 10.5 in presence of 20mg/ml ethanolic extract of *Z. officinale* [39]. Another study also reported the antibiofilm abilities of *Betula pendula*, *Equisetum arvense*, *Herniaria glabra*, *Galium odoratum*, *Urtica dioica* and *Vaccinium Vitis-idaea* extracts. In addition, the *B. pendula* and *U. dioica* extracts significantly reduced the motility of the *E. coli* rods in their study [40].

Bioautography

The bioautograms for solvent system 2 (Toluene: Methanol: Ethyl acetate) & solvent system 3 (Butanol: Acetone: Water) showed a zone of inhibition indicating the successful separation of bioactive component. The Rf values of both the solvent system were measured as 0.875. The bioautogram for solvent system 1 did not show any zone of inhibition in our study.

Bioautography is a qualitative study that enables the researcher to easily separate the bioactive components in the plant material and study them, thus making it a protocol of choice. A study carried out using extracts of 13 Brazilian medicinal plants reported their antimicrobial activities against bacteria and yeasts. They demonstrated the antibacterial compound(s) with *Rf* of 0.68 to be most effective against test pathogens. Also, the antibacterial compounds present in the extracts from *Arctium lappa* showed antibacterial activity at similar *Rf* values ranging from 0.85-0.93 [41].

GC-MS analysis of *M. fragrans* oil

The chromatogram showing Retention Time (RTm) of several constituents identified with the help of GC-MS analysis of *M. fragrans* oil is shown in Figure 1. It showed the presence of 16 peaks out of which 8 corresponded to compounds with known anti-microbial properties. The retention times and corresponding constituents of *M. fragrans* essential oil are listed in Table 5.

Table 5: Biocomponents identified from GC-MS analysis

Sr. no.	RT_m in min	Peak area	IUPAC name	Nature of compound	Biological activity
1	3.26	2×10^9	α – pinene	Terpene	Antibacterial
2	3.99	0.9×10^9	ß- pinene	Terpene	Antibacterial
3	4.44	0.5×10^9	1,3-cyclohexadine, 1-methyl-4-(1-methylethyl)-	Monoterpene	Fungistatic
4	5.63	0.2×10^9	4 – careen	Monoterpene	Antibacterial
5	7.28	0.7×10^9	3-cyclohexen-1-ol,4-methyl-1-(1-methylethyl)-	Monoterpene Oxygenated	Antimicrobial Anti-inflammatory
6	9.22	0.1×10^9	1,3-benzodioxole,5-(2-propyl)-	Aromatic oxygenated	Immune modulator
7	13.05	0.2×10^9	3-benzodioxole,4-methoxy-6-(2-propenyl)-	Aromatic oxygenated	Anti-cancer
8	13.49	0.3×10^9	Benzene-1,2,3-trimethoxy-5-(2-propenyl)-	Aromatic oxygenated	Anti-fungal Anti-viral

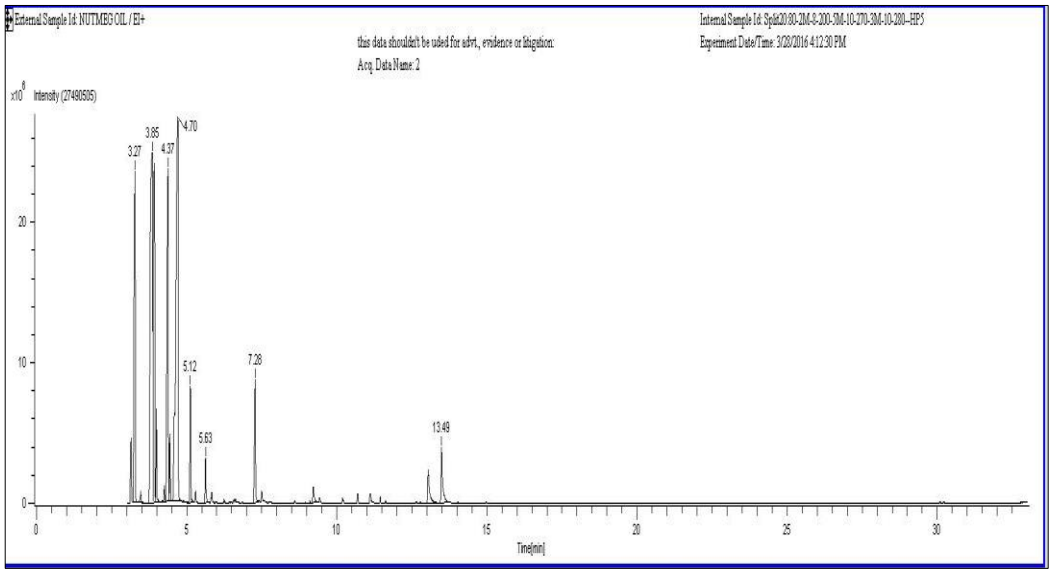

Figure 1: GC-MS chromatogram of *M. fragrans* oil

Similar to our findings, the concentration of monoterpene hydrocarbons i.e., α-pinene and β-pinene together were found to constitute around 25% volume in *M. fragrans* essential oil in a previous study [42]. Another study indicated its concentration to be as high as 41% in a freshly extracted fraction of *M. fragrans* essential oil. In addition, it also showed presence of limonene (7.5 %), myristicin (16.2 %), safrole (3.9 %) and methyl eugenol (1.8 %) [43].

CONCLUSION

The present study confirms the antibacterial activity of *M. fragrans* oil against ESBL and MBL producing uro-pathogens. Further, its potency against biofilm forming test isolates can be exploited in therapeutic formulations for the treatment of infections caused by drug-resistant pathogens.

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