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RESEARCH ARTICLE

Characterisation and Identification of Bioactive Compounds from Flower Extracts of *Myristica fragrans* and Their Pharmacological Applications

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Abstract

Background: The excessive use of antibiotics has posed a global threat and challenge to the emergence of drug resistance in pathogenic bacteria. Various innovative methods, including using naturally occurring compounds to weaken antibacterial activity, are under investigation to address this issue. Nutmeg seeds are commonly used as spices in culinary applications and traditional medicine, as they are known for their stomachic, analgesic, and stimulant properties. **Aim**: The primary objective of the present work was to assess the antimicrobial activity of the flower extract of *Myristica fragrans* post purification process by Thin Layer Chromatography (TLC) and Column Chromatography. **Methods**: The column fractions were then assessed for their antimicrobial activity against ESKAPE pathogens, followed by the characterisation of the bioactive compounds by Fourier Transform Infrared Spectroscopy (FTIR) and Gas Chromatography Mass Spectrometry (GC-MS) analysis. **Result**: The characterisation analysis resulted in a compound called elemicin, a phenyl propene compound with a high yield of 62%. The second phase of the study involved the Minimal Inhibitory Concentration (MIC) determination and biofilm inhibition of elemicin against ESKAPE pathogens, along with the toxicity study on MRC-5 cell lines with signs of no toxicity, obtaining an IC50 value of 194.889 ug/ml. **Conclusion:** This present study of the characterisation of the bioactive compounds from the flower extract of *M. fragrans* was conducted for the first time. There have been no earlier studies pertaining to this, and the results exhibited by the bioactive compound as potent biofilm inhibitors and antimicrobial agents are worth mentioning and can be further extrapolated to translational research.

Major Findings: The analytical process of characterisation of the crude extract of *M. fragrans* was a major finding in the present study where 6 different fractions were separated and each identified via GC-MS technique. The identification of 3 potent antibacterial compounds namely elemicin, p-Menth-1-en-4-ol and p-Cymene with a good yield and low cytotoxicity was a breakthrough in the study which could be further extrapolated to translational research.

Keywords: Elemicin, FTIR, GC-MS, *M. fragrans* flower, MRC-5 cell lines, MTT assay

1. Introduction

Bacteria develop defensive mechanisms to withstand the harmful effects of antibiotics, which can ultimately reduce or eliminate the antibiotics' ability to inhibit their growth. This has eventually led to the development of resistance to various available therapeutic agents, which is an alarming concern for public health worldwide. ESKAPE pathogens (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa*, and *Enterobacter* spp.) are a group of drug-resistant bacteria exhibiting multidrug resistance and virulence through various mechanisms. The terminology, "ESKAPE", was coined by the Infectious Diseases Society of America based on the data from hospital-based surveillance studies[1,2].

Myristica fragrans Houtt., commonly known as nutmeg, is a plant species native to Indonesia, primarily found in coastal and tropical regions, and

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widely recognised for its use in spice production[3]. The phytochemical constituents of nutmeg consist of essential oil, triterpenes, and various types of phenolic compounds[4], thereby contributing to various antioxidant, antimicrobial and central nervous system applications. Nutmeg Essential Oils (NEO) are rich in monoterpenes (about 90%) and phenylpropanoids. The monoterpenes that are commonly identified in NEO are α-pinene, β-pinene, 4-terpineol, α-terpinene, γ-terpinene, limonene, sabinene, α-terpineol, camphene, and myrcene, whereas the phenylpropanoid compounds that can be found are myristicin, elemicin, safrole, eugenol, methyl eugenol, and methyl isoeugenol[5].

Much of the studies of *M. fragrans* are reported with the seed, leaves and fruit parts of the plant. There has been only one study with the male flower of *M. fragrans,* which has shown excellent activity with the crude extract of the flower against foodborne pathogens. This recent study with the flower extract of *M. fragrans*[6] provided an insight to explore the bioactive compounds in the flower extract of nutmeg and carry out toxicity testing with the bioactive compound to carry out necessary work for effective translational research and find an effective drug against drug-resistant pathogens.

2. Materials and Methods

2.1 Purification of *M. Fragrans* Flower Crude Extract and its Antibacterial Activity

The dried and ground plant material was subjected to Soxhlet extraction using 100% ethanol, followed by concentration[6].

2.1.1 Thin layer Chromatography

The crude flower extracts were analysed using TLC on a commercially available 20cm × 20cm silica TLC plate (GF 25460; Merck, 250 mm thick) following the procedure outlined by Thomas and Tirumale[7]. The best solvent system (Hexane: Diethyl ether [8:2]) capable of showing maximum resolution following elution were selected under the laboratory conditions of 28 ± 5 °C and relative humidity of 40-50% and Rf value noted.

2.1.2 Column Chromatography

The crude flower extracts of *M. fragrans* were subjected to Column Chromatography for fractionation on silica gel (60-120 mesh) using a gradient solvent system of hexane-diethyl ether (8:2 v/v), followed by benzene-methanol (8.5: 0.5 v/v) and finally 100% methanol in the order of increasing polarity. Following quantification of all the eluted compounds, all the fractions were screened by TLC on a silica plate (MERCK silica gel 60 PF_{254} aluminium sheets, 250 mm thick, 20 cm x 20 cm) under visible light and ultraviolet light in both short and long wavelengths[8-9].

2.1.3 Antibacterial activity by Kirby Bauer Method

The antibacterial activity of the fractionated compounds against MTCC strains of ESKAPE pathogens (*Enterococcus faecium* MTCC 13248, *Staphylococcus aureus* MTCC 740, *Klebsiella pneumoniae* MTCC 432, *Acinetobacter baumannii* MTCC 12889, *Pseudomonas aeruginosa* MTCC 1688, *Enterobacter aerogenes* MTCC 2822) was assessed by the well diffusion method[10] with ethanol as the negative control.

2.2 Characterisation of the Fractionated Compounds

GC-MS Analysis: The fraction was analysed in the Shimadzu GC-MS-138 QP2010S, containing Rxi5ms gas chromatography column (ID 0.25mm, .25um thickness), coupled to a Mass Spectrometer mass detector. The injection volume was 1ul, and the scan range was 45 – 450 atomic mass units, with a total run time of 45 minutes. Data acquistion and analyses control was carried out by labsolutions GCMS software.

2.3 Biofilm Inhibition of the Bioactive Compounds

2.3.1 Anti-biofilm Activity by Crystal Violet Assay

Plant extract was dissolved in ethanol at a concentration of 500 mg/mL. This was further to obtain test concentrations of 100, 75, 50, 25, 12.5, 6.25, 3.12 and 1.56 mg/mL. Biofilm quantification was performed following the method of Alva *et al* (2019)[11]. A 100µL sample of the diluted culture of *S. aureus* and *P. aeruginosa* strains was added to a microtiter plate and incubated at 37°C for 24 hours, followed by washing with PBS (pH 7.4). Subsequently, 125µL of a freshly prepared 0.1% crystal violet solution was added to the dried pellet and incubated for 10 minutes. After staining and washing, 200 µl of 30% acetic acid was added to the pellet and incubated for 15 minutes to dissolve the stain,

followed by transfer of a 100µl aliquot and the optical density was recorded at 600 nm using an ELISA reader (Biorad, USA). The reduction in biofilm formation in the presence of plant extracts was calculated as per cent inhibition using the formula: [(OD of control – OD of treated) / OD of control] x 100.

2.3.2 Las B Elastolytic Assay

The Elastin Congo Red assay was performed according to the protocol of Alva *et al*[11]. Cells were cultured in Lysogeny Broth (LB) at 37°C for 14 hours and then centrifuged at 10,000 rpm for 10 minutes; subsequently 50 µL aliquot of the resulting supernatant was mixed with 2mL of 10mM Na_2HPO_4 containing 30mg of Elastin Congo Red, incubated at 37°C for 14 hours, followed by centrifugation. The absorbance of the released Congo Red was measured at 495nm.

2.3.3 Pyocyanin Quantification Assay

The assay was carried out following the method of Alva *et al*[11]. Post-culturing the cells in LB broth at 37°C for 24-48 hours, the mixture was subjected to centrifugation, followed by the addition of 3 ml of chloroform to a 5ml sample of the culture supernatant. The separated lower organic phase was mixed with 2ml of 0.2 M HCl, followed by the absorbance measurement at 520 nm. The pyocyanin concentration was expressed in µg/mL of culture supernatant and calculated by multiplying the optical density by 17.072.

2.3.4 Swarming Motility Assay

A 5 µL sample of the overnight culture was inoculated onto 0.4% nutrient agar and incubated for 24 hours. The swarm diameter on the agar plates was then measured in millimeters[11].

2.4 *In Vitro* Cytotoxicity Determination of Bioactive Compound

2.4.1 MTT Assay

MRC-5 cell line procured from the National Centre for Cell Sciences (NCCS), Pune, India, was maintained in Dulbecco's modified Eagle's medium, DMEM (Sigma-Aldrich, USA). The cytotoxicity of the compound in comparison with Doxorubicin was performed by MTT Assay[7] with concentrations ranging from 200 µg, 100 µg, 50 µg, 25 µg, 12.5 µg, 6.25 µg in 500 µl of DMEM.

2.4.2 Statistical Analysis

All experiments were conducted in triplicate, and the results are presented as Mean ± SD (n=3). Statistical significance was assessed using the unpaired Student's t-test, one-way ANOVA, and Dunnett's test (SPSS Inc., version 20.0). A probability value (P) ≤ 0.05 was considered statistically significant.

3. Results and Discussion

3.1 Extraction of *Myristica fragrans* Crude Flower Extract and its Antibacterial Activity

The total yield of flower extract of *Myristica fragrans* was found to be 4.85g, followed by purification of the crude extract.

3.2 Purification and Antibacterial Activity of the Fractionated Compounds

3.2.1 Thin Layer Chromatography

The crude flower extract of *M. fragrans* was separated on a silica gel TLC plate with a standardised mobile phase of Hexane: Diethyl ether (8:2) with a separation into 7 bands under UV light.

3.2.2 Column Chromatography

The crude flower extract of *M. fragrans* (2g) was fractionated using column chromatography on silica gel with a tertiary gradient solvent system of Hexane: Diethyl ether (8:2), benzene-methanol (8.5: 0.5 v/v) and finally 100% methanol with gradually increasing the polarity to yield 06 fractions. TLC was performed with all the fractions (1-6) for confirmation.

Antibacterial activity with the fractionated compounds by Kirby Bauer method: the antimicrobial activity of all the six column fractions of the *M. fragrans* flower extract was assessed against the ESKAPE pathogens at a concentration of 100 mg/ml wherein Fraction 1 exhibited potent activity against *S. aureus, K. pneumoniae* and *P. aeruginosa* with a zone of inhibition of 22mm, 31mm,19mm respectively; Fraction 2 showed potent activity only against *K. pneumonia* with a zone of inhibition of 17mm. Considerable activity against *S. aureus* and *P. aeruginosa,* with a zone inhibition of 21mm and 26mm, respectively, was exhibited by Fraction 3, whereas Fraction 4 showed activity against *S. aureus* and *P. aeruginosa* with a zone of inhibition

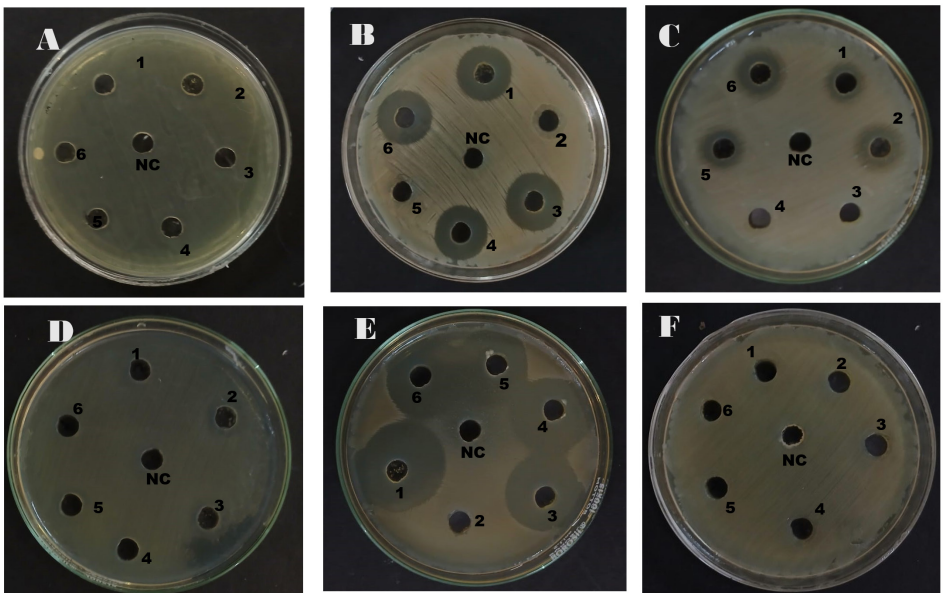


Figure 1. Antimicrobial activity of *Myristica frangrans* flower extract against; **(A).** *Enterococcus faecium,* **(B).** *Staphylococcus aureus,* **(C).** *Klebsiella pneumoniae,* **(D).** *Acinetobacter baumannii,* **(E).** *Pseudomonas aeruginosa,* **(F).** *Enterobacter aerogenes* [1-6 corresponds to the six column fractions following Chromatography; NC – Negative Control].

of 19mm and 27mm, respectively. Fraction 5 showed antibacterial activity with a zone of inhibition of 27mm and 18mm against *P. aeruginosa* and *K. pneumoniae,* and Fraction 6 showed good activity against *S. aureus* and *P. aeruginosa* and *K. pneumoniae* with a zone of inhibition of 19mm, 27mm and 16mm (Figure 1).

3.3 Characterisation and Identification of Fractionated Compounds from *M. fragrans* Flower Extract

Fraction 1, Fraction 3 and Fraction 6 were subjected for characterisation and identification by GC-MS analysis based on the antibacterial activity exhibited wherein the compounds were identified as Elemicin with a retention time of 21.21 min, molecular mass of 208 g/mol with the molecular formula $C_{12}H_{16}O_3$, p-Menth-1-en-4-ol (Retention time of 12.223 min, Molecular weight of 154 g/mol with the formula $C_{10}H_{18}O$ and p-Cymene with Molecular formula $C_{10}H_{14}O$, retention time 12.317 min and Molecular mass at 150 g/mol respectvely (Figure 2).

3.4 Anti-biofilm Activity of Fraction 1

The biofilm inhibition of *S. aureus* MTCC 740 and *P. aeruginosa* MTCC 1688 by the compound Elemicin was assessed by the Crystal Violet assay, followed by the decrease in the virulence factor of *P. aeruginosa* by pyocyanin quantification, inhibition of swarming motility and decrease in the elastase production.

3.4.1 Crystal Violet Assay

The inoculum without the extracts was taken as the control, and Tetracycline was used as the positive control. Six test concentrations of the test Fraction 1 were taken from 100 – 6.25mg/mL Similar concentrations were taken with Tetracycline as the positive control. The % of inhibition of the test compound (Table 1) and Tetracycline were determined. The IC_{50} of elemicin were found to be 55.15mg/ml and 61.23mg/ml when compared against Tetracycline with an IC 50 of 55.44mg/ml and 60.64mg/ml against *S. aureus* and *P. aeruginosa,* respectively (Figure 3A).

3.4.2 Pyocyanin Quantification

The fractionated compound from *M. fragrans* flower extract was able to inhibit the production of pyocyanin. The pigment yield of the control was calculated as 47.118 µg/ml, and the sample was found as 10.94µg/ml. There was 76.78% pigment reduction shown by the crude extract (Figure 3B).

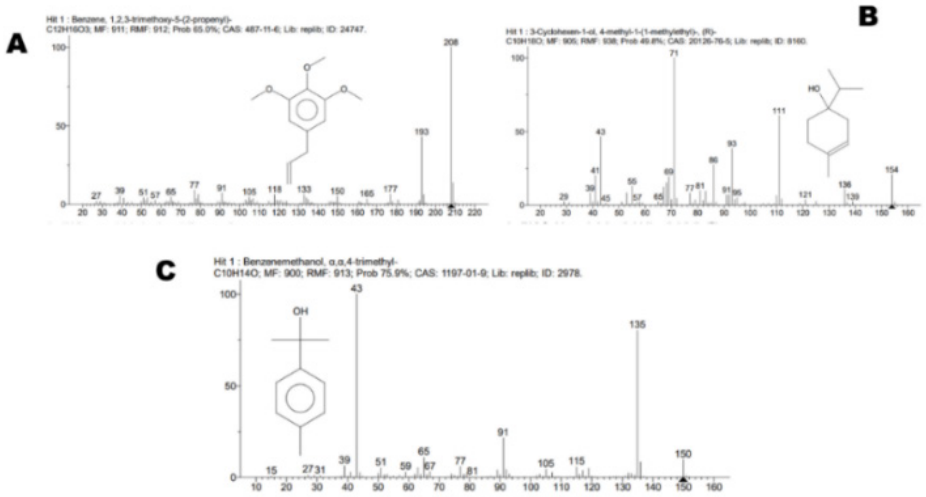


Figure 2. GC-MS analysis of Fractions 1, 3 and 6 (**A** - Fraction 1 identified as Elemicin; **B**- Fraction 3 identified as p-Menth-1-en-4-ol and **C** – Fraction 6 identified as p-Cymene).

Table 1. Biofilm inhibition of Fraction 1 and Tetracycline against test pathogens

	Concentration of Extract (mg/mL)							
	100	75	50	25	12.5	6.25	3.12	1.56
Staphylococcus aureus								
Control	1.235							
Mean ± SD	0.434 ± 0.007	0.486 ± 0.006	0.505± 0.007	0.713 ± 0.007	0.926 ± 0.008	0.996 ± 0.007	1.145 ± 0.008	1.217 ± 0.012
% of inhibition	**64.85**	**60.64**	**59.10**	**42.26**	**25.02**	**19.35**	**7.28**	**1.45**
IC 5O	**55.15 mg/ml**							
Pseudomonas aeruginosa								
Control	1.102							
Mean ± SD	0.386 ± 0.008	0.396 ± 0.009	0.480 ± 0.003	0.502 ± 0.011	0.626 ± 0.007	0.835 ± 0.008	0.888 ± 0.023	1.024 ± 0.007
% of inhibition	**64.97**	**64.06**	**56.44**	**54.44**	**43.19**	**24.22**	**19.41**	**7.07**
IC5O	**61.23 mg/ml**							

3.4.3 Swarming Motility Inhibition

There was a significant difference in the swarming motility exhibited by the control and the compound. The control showed 19mm diameter zone of inhibition and the extract showed no zone of inhibition (Figure 3C).

3.4.4 Elastase Inhibition

The elastase inhibition with the purified compound was measured at 495nm. OD value of the control was found to be 0.355nm, and the sample was 0.301nm. There was 11.47% elastase inhibition with the compound elemicin extracted from *M. fragrans* flower (Figure 3D).

3.5 *In Vitro* Cytotoxicity Testing of Elemicin on Lung Fibroblast Cell Line

3.5.1 MTT Assay

The cytotoxicity of the purified compound, elemicin, was assessed on normal human lung fibroblast cell lines (MRC-5), with doxorubicin serving as the positive control. The MTT assay indicated that elemicin had minimal toxicity, with an IC_{50} value of 194.889 µg/mL

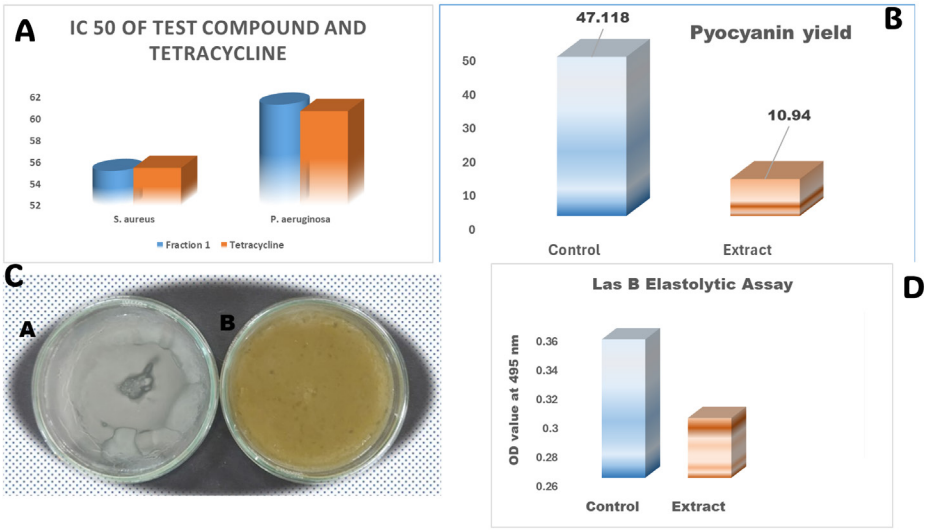


Figure 3. **(A).** IC_{50} of biofilm inhibition of fraction 1 and Tetracycline against *S. aureus* and *P. aeruginosa;* **(B).** Pyocyanin Reduction Assay of fraction 1 against *P. aeruginosa;* **(C).** Swarming motility assay of the crude extract against *P. aeruginosa* (A -Control, B- Extract); **(D).** Elastase inhibition against *P. aeruginosa*.

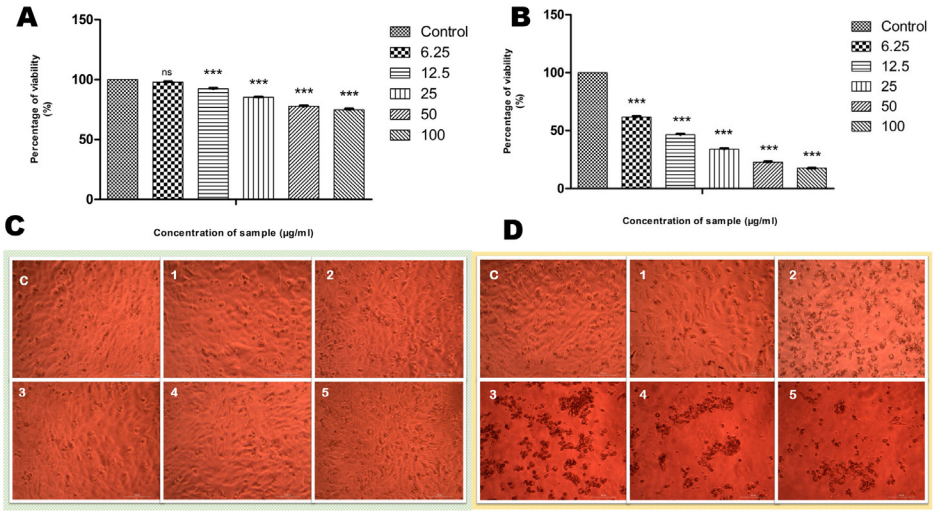


Figure 4. Graphical representation depicting the cytotoxicity effect of **(A).** crude extract of *M. fragrans;* **(B).** doxorubicin on MRC-5 cell line by MTT assay - Along Y axis percentage viability, Along X axis varied concentration of samples; Microscopic examination of cytotoxicity of **(C).** elemicin and **(D).** doxorubicin on MRC-5 cell lines under 10X magnification in inverted phase contrast microscope.
[C- Untreated control cells exhibiting epithelial morphology, 1- 6.25 µg/mL, 2- 12.5 µg/mL, 3- 25 µg/mL, 4- 50 µg/mL, 5- 100 µg/mL].

in comparison with doxorubicin with IC_{50} 11.0231 µg/mL (Figure 4 A, B, C and D).

4. Discussion

The infections caused by the ESKAPE (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa,* and *Enterobacter* species) pathogens are a significant global threat to human health, and a majority of them are resistant to antimicrobial agents, posing serious therapeutic dilemmas for physicians. Over the years of research, there have been various advanced and improved techniques in the field of genetics

and molecular biology which provide detailed information about individual resistance mechanisms in all Multidrug-Resistant (MDR) pathogens. However, there remains a lack of compelling data on their specific resistance mechanism. The previous studies[6] also cite the potent antibacterial activity against foodborne pathogens by the flower extract, though numerous activities were reported from the seeds, fruits and leaves of *M. fragrans*[12-14].

For this, the present study aimed to characterise various bioactive compounds from the flower extract of *M. fragrans,* which has not yet been researched. The samples were subjected to Soxhlet extraction with ethanol as cited by the studies by Jose *et al*[6]. Following the extraction, the best solvent system capable of showing good resolution and separation of compounds was identified, following which the bioactive compounds were eluted via Column Chromatography. The TLC of seeds, fruit, and leaves of *M. fragrans* are well defined in the previous studies, but the aerial part of *M. fragrans* was a special scenario in the research field. All six fractionated compounds obtained via Column Chromatography were subjected to antibacterial analysis against ESKAPE pathogens.

Previous studies have reported the presence of Myristicin, terpenes, Sabinene, eugenol, α-pinene, limonene, copaene, β-phellandrene, γ-Terpinene[15]. The present characterisation technique by GC-MS could identify 3 bioactive compounds exhibiting potent antibacterial activity; Fraction 1 identified as elemicin, fraction 3 as p-Menth-1-en-4-ol and fraction 6 as p-Cymene. Among these compounds, p-meth-1-en-4-ol is reported to exhibit antiparasitic properties and also antimicrobial properties[16], Antiviral and neuroprotective antitumor properties shown by p-cymen-8-ol[17] and elemicin exhibit antidepressant as well as antimicrobial and antiviral properties[18]. The biofilm inhibiting activity of elemicin against biofilm producers like *S. aureus* and *P. aeruginosa* also provides a limelight on the futuristic potential of this compound as an effective antimicrobial agent, which was researched in the present study. The future perspective of the study lies in analysing the mechanism of action of these bioactive compounds against various pathogens so that it can be converted into translational research and find a suitable non-toxic compound to replace the resistant antibiotics.

5. Conclusion

The present study was carried out to characterise and identify the bioactive compounds from the crude flower extract of *M. fragrans*. Initially, the crude extract was obtained by Soxhlet extraction using absolute alcohol as the solvent. Following experiments with various combinations of solvent systems, the best solvent system capable of good separation of compounds on the TLC plate was identified as hexane: diethyl ether (8:2 v/v), with distinct compounds visible under UV light. The purification process was carried out by column chromatography, wherein a total of 2g of the flower extract was separated on silica gel using the same solvent system, obtaining 6 separate fractions; each fraction was subjected to antimicrobial activity against the ESKAPE pathogens, where Fractions 1, 3 and 6 showed potent activities. Fractions 1 and 6 were more active against the pathogens, mainly *S. aureus*, *K. pneumoniae* and *P. aeruginosa,* whereas Fraction 3 showed potent activity against *S. aureus* and *P. aeruginosa*. The characterisation and identification of all 3 fractions were subjected to GC-MS analysis in the Shimadzu GC-MS-138 QP2010S, where the bioactive compounds were identified as elemicin, p-Menth-1-en-4-ol and p-Cymene, respectively.

The activity of elemicin as an anti-quorum sensing compound against *S. aureus* and *P. aeruginosa* was studied by biofilm inhibition activity by Crystal violet assay, decrease in the virulence factor of *P. aeruginosa* by searming motility assay, Las B elastase assay and inhibition of pyocyanin production. The IC_{50} of elemicin in inhibiting the biofilm production of *S. aureus* and *P. aeruginosa* was 55.15 mg/ml and 6.23 mg/ml against Tetracycline, having IC_{50} of 55.44 mg/ml and 60.64 mg/ml, respectively. Further, elemicin was also found to inhibit the production of pyocyanin by 76.78 %, along with inhibiting the elastase production and swarming motility.

This present study of the characterisation of the bioactive compounds from the flower extract of *M. fragrans* was conducted for the first time, and there have been no earlier studies about this. The results exhibited by the bioactive compound as a potent biofilm inhibitor and antimicrobial agent are worth mentioning and can be further extrapolated to translational research.

6. Author Contribution

The first author, Vishnupriya, was involved in Data acquisition, data analysis and interpretation and statistical analysis. The second author contributed towards the execution of the analytical study, design and framework and data analysis. The corresponding author, Tessy Anu Thomas, was involved in the experimental design, drafting the manuscript and critical revision of the manuscript. All authors have read and agreed to the published version of the manuscript.

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