RESEARCH ARTICLE

Evaluating Secondary Metabolites from Extracts of *Mimusops elengi* to Assess its Antimicrobial Activity Against Human Pathogens

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Abstract

An investigation was conducted to determine the possible phytochemical components from the methanolic, ethanolic and chloroform extracts of *Mimusops elengi*. Among the phytochemical screening of these extracts, methanolic extract showed that the whole plant was rich in alkaloids, flavonoids, glycosides, phenolic compounds and saponins. The study was extended by analysing the potent bioactive compounds in various extracts of *Mimusops elengi* using GC/MS and analyzing their functional groups using FTIR to establish the antibacterial and antifungal properties of the plant extract against bacterial (*Staphylococcus aureus* and *Escherichia coli*) and fungal pathogens (*Candida albicans*), for which the *M. elengi* plant has been significantly used as a multidrug constituent. The major compounds identified by GC/MS were cis pinen-3-ol, undecanoic acid, 3 carene, citronellol, hexadecenoic acid, caryophyllene, dodecanedioic acid and vitamin A aldehyde. These compounds have different therapeutic and antimicrobial effects. The methanolic extracts of the plant were found to be more effective against these two bacteria (*S. aureus* and *E. coli*) and fungi (*C. albicans*) compared with the ethanolic and chloroform extracts. The findings and results of this paper could help to evaluate and assess the therapeutic multipurpose use of *M. elengi* more rationally and can create an awareness of the need for in situ conservation of this most wanted medicinal plant.

Keywords: *Mimusops elengi*, Phytochemicals, GC-MS, FTIR, Antimicrobial activity, Minimum inhibitory concentration

Highlights

- *Mimusops elengi* can be considered as a therapeutic plant.
- The major compounds identified by GC/MS were cis pinen-3-ol, undecanoic acid, 3 carene, citronellol, hexadecenoic acid, caryophyllene, dodecanedioic acid and vitamin A aldehyde.
- Various phytochemical classes, such as terpenoids, saponins, glycosides, alkaloids, and phenols are present in plant parts.
- The methanolic extract of the plant parts was the most effective among the ethanolic and chloroform extracts.

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Introduction

Medicinal plants yield a broad array of secondary metabolites, many of which possess antimicrobial activities against some bacteria and fungi. Some of these secondary metabolites exist in plants and elicit therapeutic effect against various microbial infections (Akhtar *et al.,* 2014). Because antibiotic resistance against pathogenic microorganisms has developed, new antimicrobials have been derived from plant extracts. Work has been conducted to isolate bioactive compounds from plant extracts with novel mechanisms of action (Mustafa *et al*., 2017). Numerous secondary metabolites possess antimicrobial properties and are a source of potent antimicrobial agents (Aiyegoro *et al*.2009). Nowadays bacterial and fungal infections represent an important cause of death around the globe. Therefore, the development and discovery of antimicrobial drugs from natural origin for the treatment of diseases are of great importance (Maruti *et al*., 2011). Plant extracts with antimicrobial properties are highly therapeutic (Uniyal *et al*., 2006). A Number of studies in last year have been conducted to assess the efficacy of plant extracts against bacterial and fungal infections. Nowadays, curative and aromatic plants are of great importance for maintaining good health (Adegbola *et al*., 2017). The traditional use of rural herbal remedies has been found to be effective against microorganisms. Plants play a noteworthy role in treating diseases, reducing conventional treatment uses such as antibiotics (Padma *et al*., 2019). The World Health Organization (WHO) estimated that developing countries still rely on traditional medicine, which 80% of the population uses.

Medicinal plants mainly cure diseases and relieve pain and ache (Oladejo *et al.,* 2018). Medicinal plants have mainly been acknowledged because of the rising resistance of associated drug in microorganisms (Rajkumar *et al*., 2018). The medicinal properties of plants are attributed to their antibacterial, antifungal and antipyretic effects because of their

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phytochemicals. There are many medicinal plants that should be investigated for a better understanding of their therapeutic properties. However, numerous tropical plants are yet to be studied in detail for their secondary metabolite constituents. Therefore, studying more tropical medicinal plants for their therapeutic properties is necessary.

However, as Darwin proposed, nature has given every living organism a remarkable capacity to fight for survival under unfavorable conditions. This holds true for microorganisms. With the introduction of newer antibiotics in clinical practice, resistance to them also evolves in microorganisms. Over the past two decades, many reports have indicated the emergence of resistance in bacteria and fungi, to not only single but also multiple antibiotics. The therapeutic properties of plants are mainly attributed to their various phytochemical classes. The phytochemical classes include flavonoids, alkaloids, phenolics, tannins, saponins, steroids, glycosides, and terpenoids in various plant parts (Koche et al., 2016; Reddy *et al*., 2007). Secondary metabolites produced by plants have turned into medicines that people can use to treat various diseases (Vaou *et al*., 2021).

For the synthesis of useful drugs, medicinal plant parts contain substances that form them and serve as an important principle for modern drug discovery. Natural antimicrobials are nowadays a top priority because of their benefits to human health. Approximately 80% of the world's inhabitants still trust ethnobotanical medicines for mankind, most of which are developed using plant parts (Koche *et al*., 2016).

The main reason behind this is that herbal medicines are safe and do not possess any side effects (Pal *et al*., 2003). In last few years, numerous studies have been performed to check the efficacy of plant extracts against numerous pathogens. Therefore, concern for developing plant-derived products has increased (Chanda *et al*., 2011).

This hypothesis focuses on screening secondary metabolites or phytochemical classes and evaluation of bioactive compounds and their antimicrobial properties isolated from ethanol, methanol and chloroform extracts of *Mimusops elengi* by GC/MS along with FTIR profiling, which may be responsible for the plant acting as a therapeutic. The main objective of this study was to evaluate the antimicrobial activity of *M. elengi* against pathogens and to identify the respective compounds using GC/MS.

Methodology

Preparation of plant extracts and its instrumental analysis

The subject became more fascinating when considering the distribution of forest areas in and around the Amravati district. The Amravati district of Maharashtra is situated in the western part of the Vidarbha region, where many medicinal plants belonging to more than 100 different families inhabit this area. Whole plants of *M. elengi,* including leaves, bark, stem and flowers, were collected from Amravati district. The plant parts were dried, and dry powder was prepared by grinding after drying for seven days. The prepared powder (500 gm) was extracted with 100 ml of methanol, ethanol and chloroform (80%) using the soxhlet method. The extracted portions of the ethanolic, methanolic and chloroform extracts were filtered, and a hot air oven was used to evaporate the solvent. Using GC-MS, the filtrate was stored in air containers and preserved in a deep freezer to study the phytochemical classes and compounds responsible for antimicrobial properties. Microbial bioassay to be performed using the agar well diffusion assay.

Quantitative determination of phytochemicals

Phytochemical examination of an ethanolic, methanolic and chloroform extract of *M. elengi* plant extracts was performed to determine the bioactive classes, namely alkaloids, flavonoids, phenols, saponins, tannins, terpenoids, phytosterols and phlorotannins, using standard methods.

GC/MS analysis of plant's extracts

Gas chromatography analysis of ethanol, methanol, and chloroform extracts of *M. elengi* was performed on GCMS -QP ultra possessing a capillary column. The carrier gas used was Helium. One µL of methanol, ethanol, and chloroform extract was injected into the Gas Chromatography. Mass spectra in the range of 30 to 600 nm were recorded. The compounds were identified using the NIST library by comparison with mass spectra.

Gas chromatography-mass spectrophotometer (GC-MS) analysis was performed with the help of gas chromatography equipped and coupled to a mass detector for the identification of chemical constituents. The stored database in the spectrometer was compared with the fragmentation pattern of mass spectra.

Identification of compounds

The GC-MS mass spectrum was interpreted with the help of the National Institute of Standards and Technology.

FTIR

Fourier transform infrared spectroscopy (FTIR) is used to identify various types of functional groups in compounds. Dry powder extracted from each plant material using various solvents was used for FTIR analysis.10 mg of dry plant extract powder was crushed with 100 mg of potassium bromide pellet in the sample disc. FTIR analysed the crushed portion of each plant extract with a scan array of 4000 to 400 cm^{-1}.

Preparation of plant extracts for antimicrobial activity

Whole plants of *M. elengi,* including leaves, bark, stem and flowers, were collected from the Amravati district. The plant parts were prepared by cleaning and drying in shade for 7 days and then fine powder was prepared from the whole plant by grinding. Dry powder extraction (500 gm) was performed with 100 ml of ethanol, methanol and chloroform (80%) using the Soxhlet method. The extraction portion of ethanolic, methanolic and chloroform was then sieved and kept in a hot air oven to evaporate the solvents used. The leftovers were stored in air containers and kept in a deep freeze. Plant extracts that showed the presence of different classes of secondary metabolites with the help of physical, chemical and instrumental analysis were screened and later on plates were used to check its antimicrobial efficacy by agar well diffusion assay and after that, analysis of minimum inhibitory concentration done to know its minimum required quantity to inhibit the pathogens.

Collection of bacterial and fungal samples and antibiotics associated

The isolates included bacteria (*E. coli, S.aureus*), and fungus (*Candida albicans*), which were procured from the National Culture of Industrial Microorganisms, Pune. After receiving the cultures, gram staining and biochemical characterization were performed. After confirmation through biochemical tests, subculturing was performed, and the inoculum was prepared for bacterial and fungal culture.

Inoculum standardization

Bacterial and fungal suspensions corresponding to the turbidity of 0.5 McFarland standards were prepared. The resulting culture was then used as the inoculum in the study using the well diffusion assay method.

Agar well diffusion method

The Agar well-diffusion method was used to determine the antibacterial and antifungal activity. Muller Hinton agar (MHA) and nutrient agar plates were mopped using sterilized cotton wipes impregnated with 24 hours old culture and with count having 100 cfu/mL of bacteria and for fungi 48 hours old culture of 50 cfu/mL culture was used. Antibiotics such as clindamycin and vancomycin were used for bacterial culture, fluconazole for fungal culture, and 1-mg/mL concentrations were prepared to compare susceptibility with the plant extracts.

Four Wells (6 mm diameter and about 2 cm a part) were made with the help of a sterile cork borer for *M. elengi* extracts. Stock solutions of each plant extract were prepared for different agglomerations (10, 5, 2.5, 1.25 mg/mL) in different solvents such as methanol, ethanol and chloroform. Plant solvent extracts of about 100 µL of varying concentration were added into the wells and kept at room temperature for 2 hours to diffuse. Control experiments were conducted to check the growth promotion of the organisms in the absence of plant extracts. The plates were incubated at 18 to 24 hours for bacteria at 37°C and for 48 hours at 22°C for fungi. The diameter of the inhibition zone (mm) was measured.

To assess its comparability with standard drugs, solutions of vancomycin, clindamycin and fluconazole were obtained by dissolving in Muller Hinton broth. Three antibiotics of different ranges 10, 5, 2.5 and 1.25 all concentrations were prepared in mg/mL and an antimicrobial test was done against pathogens by measuring the zone of inhibition.

After assessing the antimicrobial action of plant extracts, plant extracts with different concentrations were used as minimum inhibitory concentration (MIC).

Minimum inhibitory concentration determination (MIC)

The fractions that showed antibacterial and antifungal activity were further assessed for the minimum inhibitory concentration, which is demarcated as the minimal concentration of plant extracts that constrain bacterial and fungal growth. (1-mg/mL) The stock solution was primed by liquefying 100 mg of dried plant extracts each in 5 ml of methanol, ethanol and chloroform. From this reference, 2-fold serial dilutions were prepared with different agglomerations of 10, 5, 2.5, 1.25 and 0.625 mg/mL. The concentration of plant-derived extracts was used to determine the MIC.

Broth dilution methodology was used to calculate the minimum inhibitory concentration (MIC). Briefly, 2 mL of Muller Hinton and nutrient broth were poured into six test tubes and 0.1 mL of each extract of the required concentration, which was prepared, was added to the nutrient broth and Muller Hinton broth for bacterial and fungal pathogens, respectively. Next, 0.1 mL of the inoculum of bacteria and fungi was poured into the test tube containing the extract and broth. All test tubes were properly closed and incubated for 24 hours at 37°C for bacteria and at 22°C for 48 hours for fungi. Varying visible growth was detected. The minimum inhibitory concentration was considered as the minimal concentration without the evolution of organisms. The minimum bactericidal and minimum fungicidal concentrations were assessed to determine the quantity of drug required to kill bacteria and fungi growth, respectively. For each batch, two control tubes were maintained. The two tubes contained extract without inoculum and the other contained growth medium and inoculum.

Statistical Analysis

Statistical analysis of the experimental data was performed using WASP 2.0.

Results and Discussions

This antimicrobial action may be attributed to the occurrence of phytochemical classes, namely alkaloids, steroids, glycosides, terpenoids, saponins and flavonoids because these secondary metabolites were detected in the extracts as shown in Table 1. This finding is supported by the work of (Manso *et al*., 2022), who reported that many bioactive classes, such as flavonoids, glycosides, terpenoids, saponins and alkaloids, have been recognized in *M. elengi*. Saponins contained in *M. elengi* exhibit inhibition properties because they possess surfactant properties that are polar in occurrence. As saponins diffuse into the cell membranes, they affect the constituents required by the microorganism, disrupt and finally, the cell membrane undergoes lysis and break (Bastian *et al*., 2019).

The main compounds isolated from *M. elengi* were alpha phellandrene, 3 carene, citronella, hexadecenoic acid and dodecane 2 methyl, as given in Table 2, which is in accordance with the study performed by Azhagumurugan and Rajan (Rajan *et al*., 2014) along with its IR spectrum showing characteristics

Table 1: Qualitative analysis of Phytochemicals present in extracts of *Mimusops elengi*

Plants extracts	*Alkaloids*	*Terpenoids*	*Coumarin*	*Tannin*	*Flavonoid*	*Phenol*	*Cardiac glycosides*	*saponins*
Ethanol extract	+	+	-	+	+	-	+	+
Methanol extract	+	+	-	-	+	+	+	+
Chloroform extract	-	-	-	-	+	-	+	+

Table 2: Compounds identified from *M. elengi* using different solvents

S. No.	*Methanol extract*	*Ethanol extract*	*Chloroform extract*
1	Dodecene,2 methyl	Cis pinen-3-ol	Arachidonic acid,trimethylsilylester
2	Cis chloro-9-octadecene	Citronellol	Pyrroliphene
3	Hexadecanoic acid cyclohexyl ester	Alpha-phellandrene	Hexadecane 1-1- dimethoxy
4	Cyclododecanol	3 Carene	Caryophyllene
5	Pyridine	Beta phellandrene	Vitamin A aldehyde
6	Alpha-phellandrene	Undecanoic acid	Kauren-18-ol, acetate
7	3 Carene	9,12-octadecadienoic acid	-
8	Beta phellandrene	Dodecanedioic acid,bis ester	-
9	Hexadecenoic acid	-	-
10	Dodecane-1 chloro	-	-
11	Dodecanedioic acid,bis ester	-	-

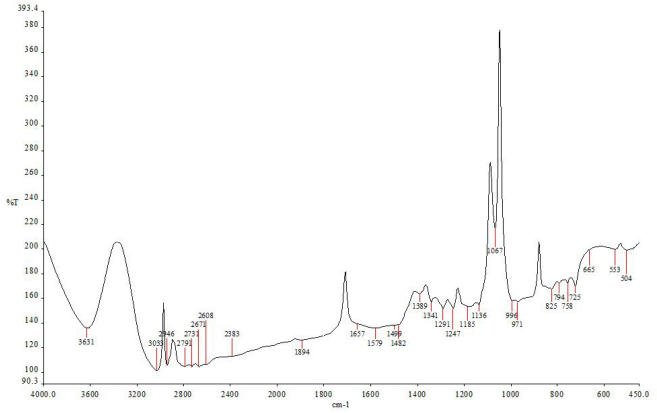

Fig. 1: FTIR graph depicting Extraction with ethanol solvent and identification of classes present in *M.elengi*

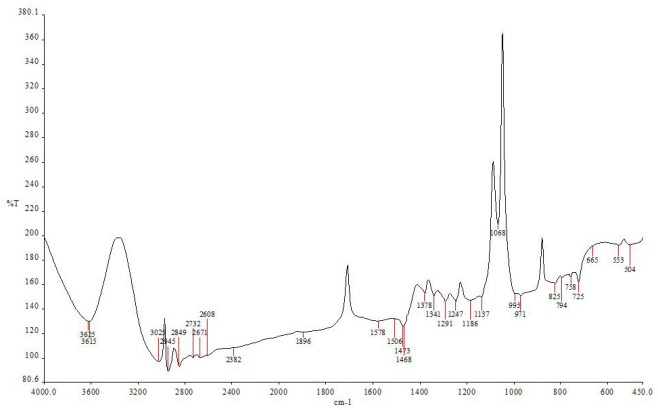

Fig. 2: FTIR graph showing Extraction with methanol solvent and identification of classes present in *M.elengi*

absorption band for hydroxyl, carbonyl, aromatic, alkene, ether and amine functional groups, which is similar to the results obtained by Biswal *et al.* (2021).

Based on the isolation method, colony and cultural characteristics, and biochemical properties, the isolates were confirmed to be *Staphylococcus aureus, Escherichia coli* and *C. albicans*. In this investigation, the methanol, ethanol, and chloroform extracts of *M. elengi* plant demonstrated antibacterial and antifungal action against three organisms *Escherichia coli, Staphylococcus aureus,* and *C. albicans*.

FTIR was used to identify the functional groups of various compounds based on the wavenumber obtained. The FTIR spectrum of the *M. elengi* plant portion in the form of KBr pellets is shown in Figs 1-3. The absorption at 3200 to 3700 is due to O-H stretching present in the extract. The band observed at 3500 cm^{-1} is attributed for primary amine stretching. The band at 2500 to 3300 cm^{-1} is assigned to carboxylic acid elongating. The band at 2800 to 3000 cm^{-1} is due to amine salt N-H elongating. The absorption band at 3000 to 3100 cm^{-1} is due to alkene C-H stretching. A notable band 2600 to 2830 cm^{-1} can be attributed to aldehyde C-H stretching. The band at 2100 to 2140 cm^{-1} is due to alkyne stretching. A band at 2150 represents ketene

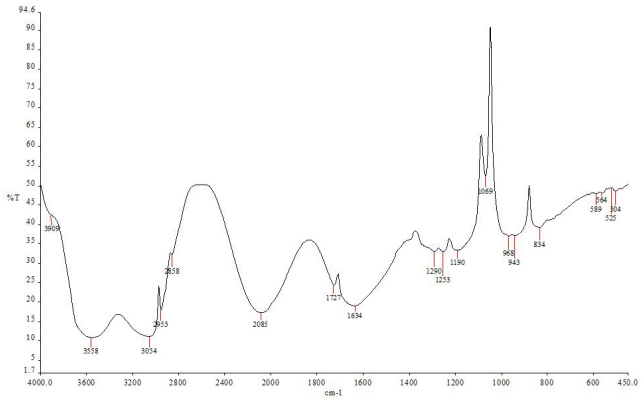

Fig. 3: FTIR graph showing Extraction with chloroform solvent and identification of classes present in *M.elengi*

C=C=O stretching. A band at 1735 to 1750 cm^{-1} is attributed to C=O stretching. The band at 1720 to 1740 cm^{-1} is attributed to aldehyde C=O stretching. The band at 1600 to 1678 cm^{-1} represents alkene C=C stretching. A band at 1380 to 1385 represents alkane C-H bending. The band at 1330 to 1420 is

Table 3: Structural characteristics of the *M. elengi* extract by FTIR record

Wavenumbers (cm^{-1})	*Characteristics*	*Wavenumbers (cm^{-1})*	*Characteristics*
3200- 3700	Alcohol O-H stretching	1720-1740	Aldehyde C=O stretching
3500	Primary amine N-H stretching	1600-1678	Alkene C=C stretching
2500-3300	Carboxylic acid O-H stretching	1380-1385	Alkane C-H bending
2800-3000	Amine salt N-H stretching	1330-1420	Alcohol O-H bending
3000-3100	Alkene C-H stretching	1266-1342	Aromatic amine C-N stretching
2600-2830	Aldehyde C-H stretching	960-980	Alkene C=C bending
2100-2140	Alkyne , carbodiimide	500-730	Halogen compound C-Cl stretching
2150	Ketene C=C=O stretching	665-730	Alkene C=C bending
1735-1750	Esters C=O stretching	490-620	Alkene C=C bending

Table 4: MIC, MBC and MFC performance of diverse plant extracts of *M. elengi* against pathogenic organisms

Microorganisms	*Ethanol extract*		*Methanol extract (mg/ml)*		*Chloroform extract (mg/ml)*	
	MIC	*MBC*	*MIC*	*MBC*	*MIC*	*MBC*
Escherichia coli	1.25	5.00	2.50	5.00	5.00	10.00
Staphylococcus aureus	1.25	5.00	2.50	5.00	5.00	10.00
	MIC	*MFC*	*MIC*	*MFC*	*MIC*	*MFC*
C. albicans	2.50	5.00	2.50	5.00	5.00	10.00

assigned to alcohol O-H bending. A band at 1266 to 1342 cm^{-1} represents aromatic amine C-N stretching. The band at 960 to 980 represents alkene C=C bending. The 835 to 805 cm^{-1} band is attributed to aromatic compound C-N stretching. The band at 500 to 730 cm^{-1} represents halogen compounds. A band at 665 to 730 cm^{-1} represents alkene with C=C bending. A band with 490 to 620 cm^{-1} absorption represents a halogen compound with C-I stretching. The Structural characteristics of *M. elengi* are shown in Table 3.

In this study, as represented in Table 4, the minimum inhibitory concentration of ethanol and methanol preparation ranged from 0.625 to 10 mg/mL against bacteria and fungi, where the dose required for the minimum inhibitory concentration required was 1.25 mg/mL, in contradiction to *E.coli* and *S,aureus*. This study's minimum concentration required to inhibit fungi was 2.50 mg/mL. In this study, the Minimum Bactericidal concentration was 5.00 mg/mL for ethanol and methanol extracts and 10.00 mg/ml for chloroform extract. This can be justified by the statement that a larger absorption of the extract complicates the procedure of dispersion of the ingredient into the microorganism cell wall (Bastian *et al*., 2019).

The zone of inhibition (mm) shown by *M. elengi* plant extracts using different solvents and standard antibiotic concentrations in mg/ml is depicted in Table 5.

The methanol extracts of *M. elengi* showed the highest inhibition against *E coli* and *S. aureus*, in accordance with the study conducted by Ansari and Ahmad (Ansari *et al*., 2020). The methanolic extract was more susceptible to gram-positive bacterial staining than the gram-negative bacteria, which is in accordance with the fact that an external peptidoglycan layer, which gram-positive bacteria do not have, is an effective penetrability barrier (Scherrer *et al*., 1971). The results of the antimicrobial effect show that *M. elengi* ethanol extract also has inhibitory properties. However, it was less than methanol extract which stated that the stronger antimicrobial action of methanol extracts is credited to the ability of the solvent to receive all the chemical complexes of non-polar, semi polar and polar constituents, so it is predictable that its antimicrobial capability is more active (Bastian *et al*., 2019). Extracts of 10, 5 and 2.5 mg/ml exhibited inhibition as compared to 1.25 mg/ml, which showed no inhibition against any organisms. The results showed that the zone of inhibition is based on concentration, and the results agree with the work of Uwimbabazi *et al*. (2015).

The ethanolic and methanolic portions of *M. elengi* showed more inhibition action against the three organisms than the chloroform extracts. The results were supported by Ansari and Ahmad (Ansari *et al*., 2020), who concluded that the robust extraction capacity of methanol and ethanol could have been responsible for the higher antimicrobial activity. The biologically active components in the plant could have been enhanced in the presence of methanol, as observed by (Akinnibosun *et al*., 2015). The antimicrobial potential of medicinal plant portions was likely due to the occurrence of alkaloids and flavonoids in the plant extract, which is well documented (Cuishine *et al*.,2011; Manner *et al*., 2013).

The methanol, ethanol and chloroform extracts compared with that of standard antibiotics (vancomycin, clindamycin, and fluconazole) have been reported to have a broad spectrum of activity, which is in accordance with the study carried out by Abbas and Abdul (Ali *et al*., 2008) as depicted in Table 5. Based on these results, vancomycin and clindamycin were used as standard drug for bacteria and fluconazole was used for fungi while assessing the antimicrobial effect of different plant extracts using solvents of *M. elengi*. Antibiotic screening tests

Table 5: Depicting zone of inhibition (mm) shown by *M. elengi* plant extracts using different solvents in and standard antibiotic concentration in mg/ml

Types of extracts	*Conc (mg/ml)*	*Diameter of zone of inhibition (mm)*			
		E.coli	*S. aureus*	*C.albicans*	*p < 0.001 **/0.05**
Ethanolic	10	17	13	15	**
	5	10	11	10	**
	2.5	7	6	7	**
	1.25	-	-	-	
Methanolic	10	20	20	18	**
	5	16	14	11	*
	2.5	9	9	6	*
	1.25	-	-	-	
Chloroform	10	12	14	13	**
	5	9	10	8	**
	2.5	6	7	5	**
	1.25	-	-	-	
Clindamycin (antibacterial standard drug)	10	20	19	-	*
	5	18	13	_	*
	2.5	8	9	_	*
	1.25	4	6	_	
vancomycin (antibacterial standard drug)	10	20	21	-	*
	5	17	13	-	**
	2.5	9	9	-	NS
	1.25	5	6	-	
Fluconazole (antifungal standard drug)	10	_	_	17	
	5	_	_	10	
	2.5	_	_	8	
	1.25	_	_	6	

Statistical analysis performed through WSAP- Web Agri Stat Pckage 2.0

** = Significant at 1%, * = Significant at 5% , NS= Non significant

against the organisms showed a high impact on vancomycin, clindamycin for bacteria and fluconazole for fungi. The antimicrobial action of different portions of *M. elengi* was tested against three microorganisms, mentioned in Table 5 along with a standard drug comparison.

The results obtained from this study showed that the isolates from *M. elengi* inhibited pathogenic fungi (*C. albicans*), which supports the statement of Ahmad and Ansari (Ansari *et al*., 2020). The inhibition of these fungi confirms the traditional therapeutic claims for the use of this plant for treating fungi. These conclusions reinforced the use of *Mimusops. elengi* for the control of various diseases.

Conclusion

The plant extract of *M. elengi* showed noteworthy inhibitions of clinically significant microorganisms that contain active phytochemicals classes such as alkaloids, glycosides, saponins, flavonoids and phenols. The components investigated by GC/MS are responsible for their antibacterial and antifungal properties. In addition, the methanolic plant extract was the most potent against the three organisms, and the ethanolic extract's minimum inhibitory concentration against *S.aureus* and *E. coli* was 1.25 mg/mL. Different plant extracts were also compared with standard drugs, demonstrating that novel compounds isolated from *M. elengi* are therapeutic agents comparable to the antibiotics used in this study. Hence from the above study we can conclude that *M. elengi* has therapeutic properties to produce novel drugs. *M. elengi* contains various bioactive compounds that are responsible for its antimicrobial properties. Therefore, these plants are of phytopharmaceutical importance. However, additional studies should be undertaken to assess the synergistic role of plant extracts of *M. elengi* with the antibiotics used in this study.

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Authors Contribution

Mrs Leena Das and Mr Vilas Kamble have designed the experiment and Mrs Leena Das has performed the experiment, organised the data, and prepared the manuscript. Mr Vilas Kamble has reviewed the data along with the whole manuscript.

Conflict of interest

None

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