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Antibacterial and antifungal activities of leaf and stem of *Marsilea minuta* L. against selected microbial pathogens

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ABSTRACT

Aim: The aim of the present study was to determine the antimicrobial activity of different solvent extracts of leaf and stem of *Marsilea minuta* (L.) against some selected pathogenic microorganisms. **Materials and Methods:** The antibacterial and antifungal studies were carried out by well diffusion method in the Research Laboratory, Department of Biochemistry, Government Arts College (Autonomous), Kumbakonam - 612 001, Tamil Nadu, India, between October and December 2015. Wells of 6mm diameter were punched in the agar medium and filled with different volume of extracts (50mg/ml) like 50, 75 and 100µl contains 2.5, 3.75 and 5mg of extract, respectively. The antimicrobial activity of different solvents such as aqueous, methanol, ethanol, and diethyl ether extracts of the leaf and stem of *M. minuta* at different concentrations was analyzed against selected bacterial pathogens such as *Bacillus subtilis, Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas fluorescens*, and *Streptococcus pyogenes* and the fungal pathogens such as *Aspergillus niger*, *Aspergillus flavus, Aspergillus terreus*, *Trichoderma viride*, and *Fusarium solani*. **Results:** The leaf and stem extracts showed antibacterial and antifungal activities by the formation of zone of inhibition ranging from 07 ± 0.8 to 25 ± 0.9 mm. Among the tested concentrations, 5 mg of both leaf and stem extracts showed best antibacterial and antifungal activities than other concentrations such as 2.5 and 3.75 mg. The maximum level of zone of inhibition 25 ± 0.9 mm was observed in aqueous stem extract against *K. pneumonia* whereas the minimum level of zone of inhibition 8 ± 0.4 mm was observed in the methanol leaf extract against *S. pyogenes*. The antifungal activity of tested solvent extracts of leaf showed positive results against in all fungal strains used in the present study. The aqueous leaf extracts showed maximum level of zone of inhibition 25±0.9mm against *T. viride*. The methanol stem extracts showed the minimum level of zone of inhibition 07±0.8mm against *A. terreus*. **Conclusion:** The results of this study confirmed the antimicrobial activity of leaf and stem of *M. minuta* and it may be source for the discovery of novel antimicrobial compounds.

1. INTRODUCTION

Ferns and their allies are in a major division of the plant kingdom called *Pteridophyta*, which have been appeared for millions of years. There are over 250 different genera of ferns and about 12,000 species. It has been observed that pteridophytes are not infected by microbial pathogens [1]. Medicinal plants play a key role in curing a variety of diseases. Bacteria and fungi cause severe infections in humans as well as animals. Many bacterial species are expressing resistance to commercial synthetic antimicrobial agents [2]. The development of new drugs from secondary metabolites produced by medicinal plants is more important [3]. Medicinal plants are readily available, accessible, affordable, potent, and relatively lower incidence of adverse reactions compared to modern synthetic drugs [4]. The researchers have intensified to screen the medicinal plants to provide a documented scientific backing and ultimately recommend them as novel sources of future antimicrobial agents [5].

Plants can be used as a valuable resource for the isolation of novel bioactive molecules to combat microbial diseases as they have been used since ancient times as a natural product for maintaining human health. The most important bioactive compounds from plants are alkaloids, flavonoids, tannins, and phenolics [6]. The extensive use of antibiotics is associated with several unwanted consequences such as toxicity, irritability, and hypersensitive reactions. Indiscriminate use of antibiotics may lead to the emergence of antibiotic-resistant strains.

The lower vascular plants such as mosses and ferns are used in different traditional Indian Systems of Medicine [7]. Many studies have been conducted on medicinal plants in different areas to evaluate their properties [8]. Food as Medicine is one of the basic concepts of traditional Siddha System of Indian Medicine [9]. The house hold recipes containing greens are part of Indian culture. Even today one can find preparations containing greens in the regular diet of South

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Indians. This tradition is passed through generations because of the immense medicinal properties including antibacterial, antifungal and antioxidant activities of greens [10].

Marsilea minuta (L.) belongs to the family of Marseliaceae and the genus Marsilea has 65 known species, among these nine species are most widely distributed in all over India [11]. *M. minuta* is a perennial fern with slender, rooted, creeping, branching rhizomes bearing erect leaves (sterile fronds) along their length [12]. The species of *Marsilea* are distributed in all parts of the world, but more common in the warmer parts of the world such as tropical regions of Africa and Australia. The medicinal plant *M. minuta* is usually found near the edge of ponds and channels and as a weed in wet rice fields. Marsiline is a macrocyclic ketone isolated from the leaves of *M. minuta*, and it possesses sedative and convulsant properties [13]. Marsiline has immense utility in psychopathy, diarrhea, cough, skin diseases, dyspepsia, fever, and insomnia. The leaf and stem of *M. minuta* are used as green vegetables in India [14,15].

M. minuta is considered as an important plant species in Ayurvedic System of Medicine with high medicinal value. *M. minuta* is reported to possess anti-infertility [16], antibacterial [17], anxiolytic [18], anticonvulsant and sedative [13], analgesic and anti-inflammatory [19], antidepressant [20], adaptogenic and antistress [14], hypocholesterolemic [21], and hepatoprotective [22] activities. Antimicrobial activity of the rhizome [23] and aerial part [24] of *M. minuta* has also been reported. However, there is not any study on antimicrobial activity of aqueous, methanol, ethanol, and diethyl ether extracts of the leaf and stem of *M. minuta* against selected microorganisms such as *Bacillus subtilis*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas fluorescens, Streptococcus pyogenes*, *Aspergillus niger*, *Aspergillus flavus, Aspergillus terreus*, *Trichoderma viride*, and *Fusarium solani.* Hence, the present study aimed to determine the antimicrobial activity of leaf and stem of *M. minuta* against above-mentioned selected human pathogenic microorganisms.

2. MATERIALS AND METHODS

2.1. Collection and Preparation of Plant Material

Healthy and disease-free plants of *M. minuta* were collected from natural habitats of Uppur Village, Thiruvarur District, Tamil Nadu, India, during October 2015. The collected plant was identified by Rev. Dr. S. John Britto, Director, Rapinat Herbarium and Centre for Molecular Systematics, St. Joseph's College, Tiruchirappalli, Tamil Nadu, India, and deposited in the herbarium (Voucher specimen number KG 002). The plants were washed thoroughly with running tap water to remove the soil particles and adhered debris and then finally washed with sterile distilled water. The leaf and stem of *M. minuta* were separated, dried under shade, and then ground well into powder. The powdered materials were stored in air-tight containers until the time of use.

2.2. Preparation of Different Solvent Extracts of *M. minuta*

50 g of the leaf and stem powder of *M. minuta* was soaked in 500 ml of aqueous, methanol, ethanol, and diethyl ether separately and then kept in an orbital shaker for 48 h at room temperature. After 48 h, the mixture was filtered through a clean muslin cloth, and the filtrates were filtered again through Whatman no.1 filter paper. Then, the filtrates were concentrated and dried using a rotary evaporator at 37°C till a sticky mass was obtained [25]. After evaporation, the dried extracts were stored at 4°C until the time of further use.

2.3. Microorganisms

The bacterial strains *B. subtilis* (microbial type culture collection [MTCC] 441), *E. coli* (MTCC 25922), *K. pneumonia* (MTCC 15380), *P. fluorescens* (MTCC 27853), and *S. pyogenes* (MTCC 29212) and the fungal strains *A. niger* (MTCC 281), *A. flavus* (MTCC 277), *A. terreus* (MTCC 1782), *T. viride* (MTCC 167), and *F. solani* (MTCC 350) were used in this study. The microorganisms were obtained from MTCC and Gene Bank, Chandigarh, India.

2.4. Antimicrobial Activity

The antimicrobial screening of aqueous, methanol, ethanol, and diethyl ether of leaf and stem extracts of *M. minuta* was carried out by well diffusion method as described by Cheesbrough [26]. 5% (w/v) test solution of the leaf and stem of *M. minuta* extracts was prepared by dissolving 250 mg of each solvent extract separately in 5 ml of sterile dimethyl sulfoxide (DMSO). From this, 50, 75, and 100 µl of the extracts containing 2.5, 3.75, and 5 mg, respectively, were taken for antimicrobial test. The extracts of leaf and stem of *M. minuta* were loaded in the well on preinoculated Mueller-Hinton agar plates with respective bacterial cultures and then incubated at 37°C for 24 h. Streptomycin (30 µg) was used as a positive control of *M. minuta* activity. The same procedure was followed for fungal species using potato dextrose agar medium, and the plates were incubated at 27°C for 48 h. In this antifungal study of *M. minuta*, amphotericin-B (50 µg) was used as a positive control. The solvent DMSO was used as a negative control in both antibacterial and antifungal experiments. After incubation, the diameter of zone of inhibition (mm) around the well was measured using zone reader. All the experiments were developed by triplicate.

2.5. Statistical Analysis

The observed results of this study were subjected to statistical analysis and the results were expressed as mean ± standard deviation of triplicate. The statistical significance was evaluated by analysis of variance (ANOVA) and the values were considered statistically significant at 5% level ($P < 0.05$).

3. RESULTS

The antibacterial activity of different solvents such as aqueous, methanol, ethanol, and diethyl ether leaf extracts of *M. minuta* at different concentrations such as 2.5, 3.75, and 5 mg was analyzed against *B. subtilis, E. coli, K. pneumoniae, P. fluorescens*, and *S. pyogenes* by well diffusion method. The observed results were measured as diameter of zone of inhibition (mm), and the results are presented in Table 1. Aqueous, methanol, ethanol, and diethyl ether extracts of the leaf of *M. minuta* showed antibacterial activity against selected bacterial species. The positive control streptomycin showed that zone of inhibition ranges between 25 ± 0.6 mm and 26 ± 0.4 mm against all bacteria included in the study. The antibacterial activity of different solvent extracts of leaf at various concentrations was compared with the activity of positive control. The levels of antibacterial activity 25 ± 0.8 mm diameter of zone of inhibition in the aqueous leaf extract against *B. subtilis* followed by diethyl ether leaf extract against *E. coli* 25 ± 0.7 mm, aqueous leaf extract against *P. fluorescens* 25 ± 0.5 mm, and methanol leaf extract against *B. subtilis* 24 ± 1.3 mm at the concentration of 5 mg/100 µl were observed as like as the activity of positive control. The minimum levels of zone of inhibition were observed in the diethyl ether leaf extract against *P. fluorescens* at 3.75 mg and 5 mg of concentrations as 9 ± 0.6 mm and 10 ± 0.9 mm, respectively. However, there was no zone of inhibition observed at 2.5 mg of concentration.

Table 1: Antibacterial activity of aqueous, methanol, ethanol, and diethyl ether leaf extracts of *Marsilea minuta*

Name of bacterial species	PC	Diameter of zone of inhibition (mm)											
		Aqueous extract			Methanol extract			Ethanol extract			Diethyl ether extract		
		50 µl (2.5 mg)	75 µl (3.75 mg)	100 µl (5 mg)	50 µl (2.5 mg)	75 µl (3.75 mg)	100 µl (5 mg)	50 µl (2.5 mg)	75 µl (3.75 mg)	100 µl (5 mg)	50 µl (2.5 mg)	75 µl (3.75 mg)	100 µl (5 mg)
Bacillus subtilis	25±0.8	20±0.5	25±0.2	25±0.8	08±0.8	14±0.9	24±1.3	17±0.8	22±0.9	22±0.6	12±1.8	20±1.3	23±0.9
Escherichia coli	25±0.9	15±0.8	18±0.9	22±1.1	10±0.4	14±0.9	20±0.2	20±0.6	22±0.8	23±0.9	20±0.4	22±0.8	25±0.7
Klebsiella pneumoniae	26±0.4	15±0.4	18±0.6	24±0.7	12±0.4	14±0.6	24±0.8	10±0.7	15±0.8	18±0.9	20±1.0	22±1.0	24±0.3
Pseudomonas fluorescens	25±0.6	15±0.6	20±0.8	25±0.5	11±0.5	12±0.7	15±0.8	10±0.8	15±0.9	20±1.1	-	09±0.6	10±0.9
Streptococcus pyogenes	25±0.8	17±0.6	18±0.9	20±1.0	08±0.4	18±0.6	23±0.7	15±0.4	15±0.5	22±0.6	15±0.4	17±0.7	20±1.1

Values are expressed as mean±standard deviation of triplicate. PC: Positive control (streptomycin 30 µg)

Similarly, the levels of antibacterial activity of different solvent extracts of the stem of *M. minuta* were also assessed, and the results are shown in Table 2. All bacteria included in the study showed sensitive against all tested solvent extracts of the stem of *M. minuta* except 2.5 mg concentration of ethanol stem extract against *K. pneumoniae*. *E. coli* was more sensitive to aqueous, methanol, ethanol, and diethyl ether stem extracts of *M. minuta* than other bacterial species. The antibacterial activity of different solvent extracts of the stem at various concentrations was also compared with the activity of the positive control streptomycin. The maximum levels of zone of inhibition in aqueous stem extract against *K. pneumonia* 25 ± 0.9 mm and *E. coli* 25 ± 0.8 mm, ethanol stem extract against *P. fluorescens* 25 ± 0.8 mm, methanol stem extract against *S. pyogenes* 25 ± 0.4 mm, and diethyl ether stem extract against *E. coli* 25 ± 0.3 mm were observed. The minimum levels of zone of inhibition in ethanolic stem extracts against *K. pneumonia* 10 ± 0.6 mm and methanolic stem extract against *P. fluorescens* 10 ± 1.2 mm and *E. coli* 13 ± 0.4 mm were observed. The results of antibacterial activity of the leaf and stem of *M. minuta* revealed that all bacterial species included in the study are sensitive to the extracts and it was varied based on the solvents and concentrations.

The statistical analysis showed that the levels of diameter of zone of inhibition were significantly ($P < 0.05$) varied within all tested categories such as bacterial species, parts of plant, solvents, concentrations, and their interactions. The results of the statistical analysis and significance are presented in Table 3. Among the bacterial species, *B. subtilis, E. coli, K. pneumoniae, P. fluorescens,* and *S. pyogenes*, a significant ($P < 0.05$) variation in the formation of zone of inhibition for both leaf and stem extracts of *M. minuta* was observed. The different concentrations were also exhibited significant ($P < 0.05$) variation in antibacterial activity by the formation of zone of inhibition against selected bacterial species for both leaf and stem extracts of *M. minuta*. Similarly, the aqueous, methanol, ethanol, and diethyl ether extracts showed significant ($P < 0.05$) variation in antibacterial activity by the formation of zone of inhibition against selected bacterial species for leaf and stem extracts of *M. minuta.* Among these, the aqueous and diethyl ether extracts showed similar antibacterial activity against selected bacterial species. Hence, based on the bacterial species, solvents, and concentrations, the antibacterial activity varied significantly ($P < 0.05$) for leaf and stem extracts of *M. minuta,* and the results of statistical analysis are shown in Table 4.

The antifungal activity of different solvents such as aqueous, methanol, ethanol, and diethyl ether leaf and stem extracts of *M. minuta* against *A. niger*, *A. flavus*, *A. terreus*, *T. viride*, and *F. solani* was analyzed. All fungal species included in the study showed sensitive against all tested solvent extracts of the leaf of *M. minuta*, and the results are shown in Table 5. The positive control amphotericin-B showed that zone of inhibition ranges between 22 ± 0.6 mm and 26 ± 0.5 mm against all tested fungal species. The antifungal activity of different solvent extracts of leaf at various concentrations was compared with the activity of the positive control. Similar levels of zone of inhibition 25 ± 0.9 mm in the aqueous leaf extract against *T. viride*, followed by diethyl ether leaf extract against *A. niger* 25 ± 0.6 mm, diethyl ether leaf extract against *A. terreus* 25 ± 0.5 mm and *F. solani* 25 ± 0.5 mm, ethanolic leaf extract against *F. solani* 25 ± 0.4 mm, and aqueous leaf extract against *F. solani* 25 ± 0.2 mm were observed. The minimum levels of zone of inhibition in the methanolic leaf extract against *A. terreus* 10 ± 0.5 mm and *F. solani* 10 ± 0.5 mm, aqueous leaf extract against *A. terreus* 10 ± 0.6 mm, and ethanolic leaf extract against *A. terreus* 10 ± 0.8 mm were observed.

All fungal species selected in this study showed sensitive against all tested solvent extracts of stem of *M. minuta*, and the results are presented in Table 6. The antifungal activity of different solvent extracts of the stem at various concentrations was compared with the activity of positive control amphotericin-B. Similar levels of zone of inhibition in ethanol, aqueous, methanol, and diethyl ether stem extracts against *F. solani* as 25 ± 0.2 mm, 24 ± 1.2 mm, 24 ± 0.9 mm, and 24 ± 0.8 mm, respectively, were also observed. Similarly, the aqueous stem extract showed zone of inhibition against *A. terreus* 24 ± 0.8 mm. The minimum levels of the zone of inhibition in the methanolic stem extract against *A. terreus* 7 ± 0.8 mm, diethyl ether stem extract against *A. niger* 10 ± 0.9 mm, and methanolic stem extract against *T. viride* 11 ± 0.8 mm and *A. niger* 12 ± 0.4 mm were observed. The results of antifungal activity of leaf and stem of *M. minuta* revealed that all fungal species included in the study are sensitive to the extracts, and it was varied based on the solvents and concentrations.

The statistical analysis showed that the levels of diameter of zone of inhibition were significantly ($P < 0.05$) varied within all tested categories such as fungal species, parts of plant, solvents, concentrations, and their interactions. The results of the statistical analysis and significance are presented in Table 7. Among the fungal species, *A. niger*, *A. flavus, A. terreus*, *T. viride*, and *F. solani*, a significant ($P < 0.05$) variation in the formation of zone of inhibition for both leaf and stem extracts of *M. minuta* was observed. However, *A. flavus* and *F. solani* are shown similar level of sensitivity for both leaf and stem extracts. Similarly, the different concentrations were exhibited significant ($P < 0.05$) variation in the antifungal activity by the formation of zone of inhibition against selected fungal species for both leaf and stem extracts. The aqueous, methanol, ethanol, and

Table 2: Antibacterial activity of aqueous, methanol, ethanol, and diethyl ether stem extracts of *Marsilea minuta*

Name of bacterial species	PC	Diameter of zone of inhibition (mm)											
		Aqueous extract			Methanol extract			Ethanol extract			Diethyl ether extract		
		50 µl (2.5 mg)	75 µl (3.75 mg)	100 µl (5 mg)	50 µl (2.5 mg)	75 µl (3.75 mg)	100 µl (5 mg)	50 µl (2.5 mg)	75 µl (3.75 mg)	100 µl (5 mg)	50 µl (2.5 mg)	75 µl (3.75 mg)	100 µl (5 mg)
Bacillus subtilis	25±0.8	15±0.8	16±1.0	20±1.1	15±0.4	19±0.6	22±0.8	17±0.8	22±0.8	24±0.9	17±0.6	20±0.7	22±0.8
Escherichia coli	25±0.9	20±0.4	23±0.7	25±0.8	13±0.4	20±0.6	22±0.8	20±0.6	22±0.8	23±0.9	20±0.7	21±0.8	25±0.3
Klebsiella pneumoniae	26±0.4	15±0.6	20±0.9	25±0.9	20±0.3	22±0.4	23±0.6	-	10±0.6	12±0.5	15±0.5	21±0.6	23±0.9
Pseudomonas fluorescens	25±0.6	15±0.5	16±0.7	20±0.8	10±1.2	13±1.3	17±0.2	20±0.5	20±0.7	25±0.8	17±0.9	17±1.0	20±1.1
Streptococcus pyogenes	25±0.8	15±0.6	18±0.8	20±1.4	22±1.2	24±1.3	25±0.4	15±0.4	15±0.5	22±0.6	15±0.6	22±0.7	24±0.9

Values are expressed as mean±standard deviation of triplicate. PC: Positive control (streptomycin 30 µg)

Table 3: ANOVA to test the validity of relationship between antimicrobial activity by zone of inhibition and indicated variables such as bacterial species, plant parts, solvents, and concentrations

Source	Sum of square (SS)	Degrees of freedom (Df)	Mean square (MS)	*F* value
Corrected model	10922.424	157	69.570	75.905
Intercept	187699.513	1	187699.513	204791.384
Main effects				
Bacterial species (A)	538.495	4	134.624	146.882***
Plant parts (B)	156.933	1	156.933	171.233***
Solvents (C)	179.453	3	59.818	65.265***
Concentrations (D)	6327.626	3	2109.209	2301.273***
Interactions				
A×B	93.579	4	23.395	25.525***
A×C	1057.904	12	88.159	96.186***
A×D	252.274	12	21.023	22.937***
B×C	205.517	3	68.506	74.744***
B×D	66.174	3	22.058	24.067***
C×D	150.737	9	16.749	18.274***
A×B × C	599.506	12	49.956	54.508***
A×B × D	135.772	12	11.314	12.345***
A×C × D	693.954	36	19.276	21.032***
B×C × D	169.031	9	18.781	20.491***
A×B × C×D	464.707	34	13.668	14.912***
Error	289.627	316	0.917	
Total	203561.950	474		
Corrected total	11212.051	473		

Level of significance: ***$P<0.001$

diethyl ether extracts were also shown significant ($P < 0.05$) variation in antifungal activity by the formation of zone of inhibition against selected fungal species for leaf and stem of *M. minuta.* Hence, based on the fungal species, solvents, and concentrations, the antifungal activity varied significantly ($P < 0.05$) for leaf and stem extracts of *M. minuta*, and the results of statistical analysis are shown in Table 8.

4. DISCUSSION

The synthetic antimicrobial agents have adverse and side effects, but the plant-derived compounds are generally safer and often more effective substitutes for the synthetic antimicrobial agents. The herbal medicines are believed to be much safer for use and proved as an elixir in the treatment on various illness and diseases [27]. Hence, there is a need to scientifically validate medicinally useful plants because of the appearance of drug resistance to antimicrobial agents and more effort is being made to find alternative antimicrobial components [28]. Plants are the storehouse and source of safer and cheaper chemicals which are pharmacologically active. The phytocompounds such as alkaloids, tannins, saponins, flavonoids, and phenolics protect the plants from their invaders such as fungi, bacteria, viruses, and nematodes [29]. The pteridophytic plants are being used ethnobotanically by various tribal communities. The antibacterial activity of 12 important pteridophytic plant extracts was reported [30].

In the present study, the extracts of leaf and stem of *M. minuta* showed antibacterial activity against both selected Gram-positive

Table 4: DMRT to rank the mean values of zone of inhibition (mm) based on bacterial species, solvents, and concentrations of extracts

Name of bacterial species				
Pseudomonas fluorescens	***Streptococcus pyogenes***	***Klebsiella pneumoniae***	***Bacillus subtilis***	***Escherichia coli***
18.42[a]	19.25[b]	20.00[c]	20.66[d]	21.41[e]
Name of solvent extracts				
Methanol extract	**Ethanol extract**	**Aqueous extract**		**Diethyl ether extract**
19.11[a]	20.08[b]	20.68[c]		20.72[c]
Concentrations of extracts				
50 µl	**75 µl**	**100 µl**		**PC**
15.43[a]	18.18[b]	21.98[c]		25.98[d]

Mean values with the same letters indicate no significant differences ($P<0.05$). PC: Positive control, DMRT: Duncan multiple range test

Table 5: Antifungal activity of aqueous, methanol, ethanol, and diethyl ether leaf extracts of *Marsilea minuta*

Name of fungal species	PC	Diameter of zone of inhibition (mm)											
		Aqueous extract			Methanol extract			Ethanol extract			Diethyl ether extract		
		50 µl (2.5 mg)	75 µl (3.75 mg)	100 µl (5 mg)	50 µl (2.5 mg)	75 µl (3.75 mg)	100 µl (5 mg)	50 µl (2.5 mg)	75 µl (3.75 mg)	100 µl (5 mg)	50 µl (2.5 mg)	75 µl (3.75 mg)	100 µl (5 mg)
Aspergillus niger	25±0.8	17±0.7	22±1.1	24±0.6	20±1.4	21±0.6	22±1.6	17±0.4	20±0.5	23±0.7	18±0.9	20±0.8	25±0.6
Aspergillus flavus	22±0.6	15±0.7	20±0.8	21±0.9	14±0.4	17±0.7	19±0.8	17±0.7	20±0.8	22±0.3	18±0.5	19±0.7	20±0.7
Aspergillus terreus	25±0.7	10±0.6	15±0.8	22±1.0	10±0.5	14±1.5	16±0.8	10±0.8	15±0.9	24±1.0	12±0.6	22±0.7	25±0.5
Trichoderma viride	26±0.5	15±0.9	20±1.1	25±0.9	15±0.4	22±1.3	24±1.3	15±0.8	22±1.0	23±1.1	17±0.7	21±0.8	24±0.9
Fusarium solani	25±0.8	15±1.1	18±1.3	25±0.2	10±0.5	12±0.7	17±1.2	15±0.8	18±1.3	25±0.4	20±1.1	22±1.4	25±0.5

Values are expressed as mean±standard deviation of triplicate. PC: Positive control (amphotericin-B 50 µg)

Table 6: Antifungal activity of aqueous, methanol, ethanol, and diethyl ether stem extracts of *Marsilea minuta*

Name of fungal species	PC	Diameter of zone of inhibition (mm)											
		Aqueous extract			Methanol extract			Ethanol extract			Diethyl ether extract		
		50 µl (2.5 mg)	75 µl (3.75 mg)	100 µl (5 mg)	50 µl (2.5 mg)	75 µl (3.75 mg)	100 µl (5 mg)	50 µl (2.5 mg)	75 µl (3.75 mg)	100 µl (5 mg)	50 µl (2.5 mg)	75 µl (3.75 mg)	100 µl (5 mg)
Aspergillus niger	25±0.8	17±1.0	15±1.1	22±1.2	12±0.4	16±0.2	20±0.9	15±0.5	22±0.6	23±0.7	10±0.9	22±1.1	22±1.3
Aspergillus flavus	22±0.6	15±0.6	18±0.7	20±0.8	15±0.9	20±1.2	22±0.8	15±0.1	20±1.1	22±0.3	20±0.6	22±0.5	24±0.3
Aspergillus terreus	25±0.7	15±0.6	19±0.7	24±0.8	07±0.8	17±0.9	22±1.1	16±0.6	15±0.7	20±0.8	15±0.4	20±0.5	23±0.7
Trichoderma viride	26±0.5	15±0.9	19±1.1	20±1.2	11±0.8	19±1.1	20±1.2	20±0.4	21±0.7	22±0.9	17±0.9	19±1.1	22±0.3
Fusarium solani	25±0.8	15±0.9	22±1.1	24±1.2	17±0.7	20±0.8	24±0.9	15±0.8	18±0.9	25±0.2	17±0.1	12±0.5	24±0.8

Values are expressed as mean±standard deviation of triplicate. PC: Positive control (amphotericin-B 50 µg)

bacteria *K. pneumoniae*, *E. coli*, and *P. fluorescens* and Gram-negative bacteria *B. subtilis* and *S. pyogenes*. The present study results are in accordance with the other studies, in that they reported the antimicrobial activities of leaf of *Marsilea quadrifolia* [31-33] and whole plant of *M. minuta* [34]. In our previous study, the antibacterial activity of leaf and stem of *M. quadrifolia* against *K. pneumoniae*, *E. coli*, *P. fluorescens*, *B. subtilis*, and *S. pyogenes* was reported [35]. Similarly, the *in vitro* antibacterial activities of medicinal plants against human pathogenic microbes were reported by several researchers [36-38].

The antibacterial activity of the extracts of leaf and stem of *M. minuta* was observed at varying degrees based on solvents, bacterial strains, concentrations of extract, and parts of plant. The four different solvent extracts of the leaf and stem of *M. minuta* exhibited antibacterial activity against all five tested bacterial species. However, the antibacterial activity of extracts against all five tested bacterial species was varied with the solvents. In the present study, the antimicrobial activity of leaf and stem extracts of *M. minuta* was observed as dose dependent. Because, the present study results revealed that the higher dose of extract (5 mg) exhibited higher zone of inhibition than the lower doses (2.5 mg and 3.75 mg) of extract of leaf and stem of *M. minuta*. Hence, this study proved that the different solvent extracts of leaf and stem of *M. minuta* possess different levels of antibacterial activity against selected bacterial species at different concentrations such as 2.5, 3.75, and 5 mg.

The present study showed that the antifungal activity of extracts of leaf and stem of *M. minuta* against selected fungal pathogens such as *A. niger, A. flavus, A. terreus, T. viride*, and *F. solani*. These results are consistent with other studies, in that the antimicrobial activity of rhizome and frond extracts of *M. minuta* [39] and the antibacterial and antifungal activities of the leaf, fruits, and latex of *Croton bonplandianum* [40] were reported. Thus, the broad spectrum of antimicrobial activity of extracts of leaf and stem of *M. minuta* is consistent with the previous study in that the root, flower, and leaf of *Aerva lanata* (L.) showed zone of inhibition against selected microbial pathogens [41]. There was also observed different levels of antifungal activity of leaf and stem extracts of *M. minuta* based on solvents, fungal species, concentrations, and

Table 7: ANOVA to test the validity of relationship between antimicrobial activity by zone of inhibition and indicated variables such as fungal species, plant parts, solvents, and concentrations

Source	Sum of square (SS)	Degrees of freedom (Df)	Mean square (MS)	*F* value
Corrected model	8801.478	159	55.355	48.751
Intercept	19778.408	1	195778.408	172419.99
Main effects				
Fungal species (A)	178.271	4	44.568	39.250***
Plant parts (B)	0.432	1	0.432	0.380^{NS}
Solvents (C)	340.297	3	113.432	99.898***
Concentration (D)	554.338	3	1718.113	513.117***
Interactions				
A×B	99.409	4	24.852	21.887***
A×C	117.110	12	9.759	8.595***
A×D	943.949	12	78.662	69.277***
B×C	22.594	3	6414	5.648***
B×D	19.241	3	6.414	5.648***
C×D	206.566	9	22.952	20.213***
A×B × C	452.700	12	37.725	33.224***
A×B × D	61.483	12	5.124	4.512***
A×C × D	446.705	36	12.408	10.928***
B×C × D	114.941	9	12.771	11.247***
A×B × C×D	643.442	36	7.873	15.741***
Error	363.353	320	1.135	
Total	204943.240	480		
Corrected total	9164.832	479		

Level of significance: ***P<0.001. NS: Not significant (P<0.05)

Table 8: DMRT to rank the mean values of zone of inhibition (mm) based on fungal species, solvents, and concentrations of extracts

Name of fungal species				
Aspergillus terreus	***Trichoderma viride***	***Aspergillus flavus***	***Fusarium solani***	***Aspergillus niger***
19.04^{a}	20.15^{b}	20.64^{c}	20.46^{c}	20.69^{d}
Name of solvent extracts				
Methanol extract	**Aqueous extract**	**Ethanol extract**		**Diethyl ether extract**
18.91^{a}	20.03^{b}	20.73^{c}		21.13^{d}
Concentrations of extract				
50 µl	**75 µl**	**100 µl**		**PC**
15.45^{a}	18.86^{b}	23.08^{c}		25.28^{d}

Mean values with the same letters indicate not significant differences (P<0.05). PC: Positive control, DMRT: Duncan multiple range test

parts of plant. The leaf and stem extracts exhibited antifungal activity against all fungal species selected in the study, but it was varied with the solvents. The antifungal activity of leaf and stem extracts of *M. minuta* was also dose dependent. The higher dose of extract exhibited higher zone of inhibition than the lower doses of extract of leaf and stem of *M. minuta*. Hence, the present study revealed the variation in antifungal activity of leaf and stem of *M. minuta* against selected fungal species based on concentrations and solvents of extracts.

Fungal diseases are represented as a critical problem to health, and they are one of the main causes of morbidity and mortality worldwide [42]. The present study showed that the wide spectrum antibacterial and antifungal properties of different solvent extracts of leaf and stem of *M. minuta* against selected bacterial and fungal species. Medicinal plants either individually or collectively may be effective as antimicrobials for Gram-positive as well as Gram-negative bacteria, fungi, actinomycetes, protozoa, etc. [43]. In our previous study, there are many secondary metabolites screened in the leaf and stem of *M. minuta* by GC-MS analysis including the compounds possess antimicrobial properties such as hexanal, 2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one, benzene carboxylic acid, 2,3-dihydro benzofuran, tetradecanoic acid, octanoic acid, and 10-octadecenoic acid [44]. Hence, the antibacterial and antifungal activities of leaf and stem of *M. minuta* may be due to the presence of bioactive compounds with antimicrobial properties.

The antimicrobial properties of leaf and stem extracts of *M. minuta* are of great interest in both academic research and herbal industry.

So, the use of *M. minuta* as natural food supplement may be emerged from a growing tendency towards to replace synthetic antimicrobial agents by natural ones. Hence, this study results introduce a unique natural source, which possesses strong antimicrobial substances, and this seems to confirm the traditional therapeutic claims of *M. minuta*.

5. CONCLUSION

The results confirmed that the leaf and stem of *M. minuta* possess antibacterial and antifungal activities, and this study is also concluded that it may be useful in the treatment of infectious diseases caused by bacteria and fungi. It is also hoped that this study would lead to the establishment of some bioactive compounds, which may be useful to formulate new and more potent antimicrobial agents from *M. minuta* for the treatment of bacterial and fungal diseases.

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