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Antimicrobial Activity of *Marsilea quadrifolia* (L.) Against Some Selected Pathogenic Microorganisms

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Authors' contributions

This work was carried out in collaboration between both authors. Both authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

Objective: To investigate the antimicrobial activity of different solvent extracts of leaf and stem of *M. quadrifolia* (L.) against selected human pathogenic microorganisms.
Methods: The antimicrobial activity was evaluated by well diffusion method. The antibacterial and antifungal studies were carried out at the Department Laboratory, Government Arts College (Autonomous), Kumbakonam – 612 001, Tamilnadu, India during the months of July to December 2013. Wells of 6 mm diameter were punched in the agar medium and filled with different volumes of extracts (50mg/ml) contains 2.5, 3.75 and 5mg concentrations.
Results: The antimicrobial activity of different solvent extracts of leaf and stem of *M. quadrifolia* at different concentrations was analyzed. Among the concentrations, 5mg of both leaf and stem extracts showed best antimicrobial activity than other concentrations 2.5 and 3.75mg. The leaf and stem extracts showed antimicrobial activity and produced the zone of inhibition ranges from 8 to 23mm. The aqueous leaf extract showed maximum zone of inhibition 23mm against *Streptococcus pyogenes* followed by ethanolic stem extract showed 21mm against *Bacillus subtilis*. The minimum antibacterial activity 8mm was observed by diethyl ether stem extract against *Klebsiella pneumonia.* The antifungal activity of diethyl ether leaf extract showed positive results in all tested fungal strains when compared to other solvent extracts. The maximum zone of inhibition 13mm was observed against *Aspergillus terreus* at 5mg of diethyl ether leaf extract. Aqueous and methanolic

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leaf extracts had no antifungal activity in all tested fungal strains except 5mg of methanolic leaf extract. The aqueous and diethyl ether stem extracts showed potent antifungal activity and the maximum zone of inhibition 15mm was observed against *Aspergillus niger*. Diethyl ether stem extract also showed maximum zone of inhibition 15mm against *Trichoderma viride*.

Conclusion: From this study, we concluded that it may be a new source for the discovery of novel antimicrobial compounds from *M. quadrifolia.*

Keywords: Marsilea quadrifolia; antimicrobial activity; well diffusion method; microorganisms; leaf; stem.

1. INTRODUCTION

Nature has served as a rich repository of medicinal plants for thousands of years. An impressive number of modern drugs have been isolated from natural sources, notably of plant origin [1]. Infectious diseases are the world's leading cause of premature deaths. Medicinal plants have been used to cure diseases since antiquity. In recent years, drug resistance of human pathogenic bacteria has been commonly reported from all over the world [2-4]. The World Health Organization (WHO) estimated that 80% of the population of developing countries relies on traditional medicines, mostly plant drugs for their primary health care needs [5,6]. However, the situation is alarming in developing as well as developed countries due to indiscriminate use of antibiotics. The drug resistant bacteria and fungal pathogens cause infectious diseases have further complicated to treat AIDS and cancer patients [7,8].

Plant derived substances have recently become a great interest owing to their versatile applications. Medicinal plants are the richest source for traditional systems of medicine, modern medicines, nutraceuticals, food supplements, folk medicines, pharmaceutical intermediates and chemical entities for synthetic drugs [9]. Plants still constitute one of the major sources of drug in modern as well as traditional medicine throughout the world [10].

A large number of Indian medicinal plants are regularly employed as antibiotic agents by medical practitioners of Ayurveda and Unani systems of medicine [11]. The therapeutic effects of plant materials due to presence of secondary metabolic compounds such as alkaloids, steroids, tannins, phenolic compounds, flavonoids and steroids are capable of producing definite physiological action on body [12,13].

Marsilea quadrifolia is known as an aquatic fern belongs to the family of Marsileaceae. *M. quadrifolia* is beneficial for nutrient mitigation from the fresh water lake and significant progress has been made for wetland restoration [14,15]. A juice made from the leaves is used to treat diuretic, febrifuge, snakebite and also applied to abscesses [16]. It is highly nutritious, good for reducing body heat, thirst and act as anti-inflammatory drug [17].

M. quadrifolia is also used to treat cough, bronchitis, diabetes, psychiatric diseases, eye diseases, diarrhoea and skin diseases [18,19]. As per the traditional claims, *M. quadrifolia* plant has been used for astringent, hypnotic, expectorant, aphrodisiac, anodyne, ophthalmic, constipating, strangury, dyspepsia and also used in the treatment of leprosy, haemorrhoids, fever and insomnia [20,21]. Antibacterial, cytotoxic and antioxidant activities of petroleum ether, chloroform and ethyl acetate extracts of aerial parts of *M. quadrifolia* have been reported [22]. Antimicrobial activity of leaf extract of *M. quadrifolia* against various bacterial pathogens was also reported [23]. However there is no study on antimicrobial activity of leaf

and stem of *M. quadrifolia* extracts using different solvents like aqueous, methanol, ethanol, and diethyl ether against selected microorganisms like *Bacillus subtilis, Escherichia coli, Klebsiella pneumoniae*, *Pseudomonas fluorescens, Streptococcus pyogenes, Aspergillus niger, Aspergillus flavus, Aspergillus terreus, Trichoderma viride and Fusarium solani.* So, the present study was designed to investigate the antimicrobial activity of leaf and stem of *M. quadrifolia* against selected human pathogenic microorganisms.

2. MATERIALS AND METHODS

2.1 Collection and Preparation of Plant Material

The fresh plants of *M. quadrifolia* were collected from natural habitats of Pathur Village, Tiruvarur District, Tamilnadu, India during the month of June 2013. The collected plant was identified by Rev. S. John Britto, Director, Rabinet Herbarium and Centre for Molecular Systematics, St. Josephs College, Tiruchirappalli, Tamilnadu, India and deposited in the herbarium (Voucher specimen number: KG 001). The plants were washed thoroughly in running tap water to remove soil particles and adhered debris and then finally washed with sterile distilled water. The leaf and stem of *M. quadrifolia* were separated and dried under shade then ground well into fine powder. The powdered materials were stored in air tight containers until the time of use.

2.2 Preparation of Plant Extract

50g of leaf and stem powder of *M. quadrifolia* were soaked in 500ml of aqueous, methanol, ethanol and diethyl ether individually and then kept in orbital shaker for 48h at room temperature. After 48h, the mixture was filtered through a clean muslin cloth. The filtrate again filtered by using a Whatman no. 1 filter paper and then the extracts were concentrated and dried in a rotary evaporator at 37ºC [24] till a sticky mass was obtained. After evaporation, the dried extracts were stored at 4ºC until further use.

2.3 Microorganisms

The following bacterial species like *Bacillus subtilis* (MTCC 441), *Escherichia coli* (MTCC 25922), *Klebsiella pneumoniae* (MTCC 15380), *Pseudomonas fluorescens* (MTCC 27853) and *Streptococcus pyogenes* (MTCC 29212) and fungal species *Aspergillus niger* (MTCC 281), *Aspergillus flavus* (MTCC 277), *Aspergillus terreus* (MTCC 1782), *Trichoderma viride* (MTCC 167) and *Fusarium solani* (MTCC 350) were used in this study. The microorganisms were obtained from Microbial Type Culture Collection and Gene Bank (MTCC), Chandigarh, India. The bacterial cultures were maintained in nutrient agar (NA) slants at 4ºC.

2.4 Antimicrobial Activity

The antimicrobial screening of leaf and stem extracts of *M. quadrifolia* was carried out by well diffusion method as described by Cheesbrough [25] at the Department Laboratory, Government Arts College (Autonomous), Kumbakonam – 612 001, Tamilnadu, India. 5% w/v test solution of leaf and stem of *M. quadrifolia* extracts were prepared by dissolving 250mg of each solvent extract separately in 5ml of sterile Dimethyl Sulphaoxide (DMSO). From this 50, 75 and 100µl extracts contains 2.5, 3.75 and 5mg, respectively were taken for antimicrobial test. The extracts of leaf and stem of *M. quadrifolia* were loaded at different concentrations 2.5, 3.75 and 5mg in the well on pre inoculated Mueller Hinton Agar

(MHA) plates with respective bacterial cultures and incubated at 37ºC for 24hrs. Streptomycin (30µg) was used as a positive control for bacterial species. The same procedure was followed for fungal species with respective medium and it was incubated at 37ºC for 48hrs. Amphotericin-B (50µg) was used as a positive control for fungal species and the solvent DMSO was used as negative control for both antibacterial and antifungal experiments. After incubation the diameter of zone of inhibition (mm) around the well was measured using zone reader.

2.5 Statistical Analysis

The results of the present study were subjected to statistical analysis and the results were expressed as mean ± standard deviation.

3. RESULTS

The antibacterial activity of different solvent extracts of leaf and stem of *M. quadrifolia* at different concentrations 2.5, 3.75 and 5mg were analyzed against *Bacillus subtilis, Escherichia coli, Klebsiella pneumoniae, Pseudomonas fluorescens and Streptococcus pyogenes* by well diffusion method. The observed results were measured as diameter of zone of inhibition ranges from 8 to 23mm and are showed in Table 1. Aqueous, ethanol and methanol leaf extracts were most effective than diethyl ether extract. *Klebsiella pneumoniae* was more sensitive to all solvent leaf extracts when compared to other organisms. Diethyl ether leaf extract showed low level of inhibition against the tested organisms when compared to other extracts. The maximum antibacterial activity was observed in aqueous leaf extract about 23±0.5mm against *Streptococcus pyogenes.*

Antibacterial potential of different solvent extracts of stem of *M. quadrifolia* were assessed and the results are given in Table 2. *Bacillus subtilis* and *Escherichia coli* were more sensitive to ethanol and diethyl either stem extracts. *Bacillus subtilis, Escherichia coli, Pseudomonas fluorescens and Streptococcus pyogenes* showed positive results against all tested solvent extracts of stem. Aqueous and methanolic stem extracts had no significant antibacterial activities against *Klebsiella pneumonia*. Ethanolic stem extract exhibited relatively higher zone of inhibition against *Bacillus subtilis* followed by diethyl either, methanol and aqueous extracts. All the tested solvent extracts of stem showed positive results against *Streptococcus pyogenes* when compared to other bacterial strains.

Antifungal activity of *M. quadrifolia* leaf and stem was analyzed and reported in Table 3 and 4. The diethyl either leaf extract had maximum level of zone of inhibition against *Aspergillus flavus* and *Aspergillus terreus* when compared to other solvent extracts. Aqueous and methanolic leaf extracts had no antifungal activities against all tested fungal organisms except 5 mg of methanolic extract. Aqueous and diethyl ether stem extracts showed positive results against all tested fungal organisms at different concentrations. Methanolic extract of stem showed low level of zone of inhibition at 5mg of concentration against tested fungal organisms except *Aspergillus niger*. There is no activity was found at 2.5 and 3.75mg concentrations of methanolic stem extracts against all tested fungal organisms. The Ethanolic stem extracts are also showed antifungal activity against *Trichoderma viride* and Fusarium solani in all tested concentrations. Ethanolic stem extract showed low level of antifungal activity 8±1.0 mm against *Aspergillus niger* at 5 mg of concentration. Ethanolic stem extracts also showed antifungal activity against *Aspergillus terreus* at 3.75 and 5mg concentrations like 11±0.5 and 12±1.0 mm, respectively. But there is no antifungal activity was found in ethanolic stem extracts against *Aspergillus flavus* in all tested concentrations.

Table 1. Antibacterial activity of different solvents like aqueous, methanol, ethanol and diethyl ether extracts of leaf of *M. quadrifolia*

Name of bacterial species	NC	PC	Diameter of zone of inhibition (mm)											
			Aqueous extract			Methanol extract			Ethanol extract			Diethyl ether extract		
			50µl (2.5mg)	75µl (3.75mg)	100µl (5mg)	50µl (2.5mg)	75µl (3.75mg)	100µl (5mg)	50µl (2.5mg)	75µl (3.75mg)	100µl (5mg)	50µl (2.5mg)	75µl (3.75mg)	100µl (5mg)
Bacillus subtilis	-	28±0.5	11±1.0	14±0.8	14±1.0	08±0.5	11±1.0	12±1.0	16±1.0	17±1.0	17±1.0	-	09±1.0	09±1.0
Escherichia coli	-	30±0.5	11±1.0	14±0.8	15±0.5	-	09±0.5	10±0.5	13±0.5	15±0.5	17±1.0	-	09±1.0	09±1.0
Klebsiella pneumoniae	-	26±0.5	12±0.5	15±1.0	15±0.5	10±0.5	13±0.8	15±1.0	18±1.0	20±0.5	21±1.0	08±1.0	10±0.5	11±1.0
Pseudomonas fluorescens	-	29±0.5	13±1.0	14±0.8	15±0.5	12±1.0	14±0.8	14±1.0	10±1.0	11±1.0	12±0.5	-	-	-
Streptococcus pyogenes	-	30±0.5	20±1.2	22±1.0	23±.0.5	09±0.5	16±1.0	17±1.0	-	-	09±1.0	09±1.0	10±0.5	11±0.5

Values are expressed as mean ± standard deviation of triplicates; NC - Negative control (DMSO); PC - Positive control (Streptomycin 30µg)

Table 2. Antibacterial activity of different solvents like aqueous, methanol, ethanol and diethyl ether extracts of stem of *M. quadrifolia*

Name of bacterial species	NC	PC	Diameter of zone of inhibition (mm)											
			Aqueous extract			Methanol extract			Ethanol extract			Diethyl ether extract		
			50µl (2.5mg)	75µl (3.75mg)	100µl (5mg)	50 µl (2.5mg)	75 µl (3.75mg)	100 µl (5mg)	50µl (2.5mg)	75µl (3.75mg)	100µl (5mg)	50µl (2.5mg)	75µl (3.75mg)	100µl (5mg)
Bacillus subtilis	-	28±0.5	11±1.0	13±0.5	14±1.0	08±0.5	14±1.0	17±0.9	18±0.5	20±1.0	21±0.5	20±0.5	20±0.5	20±0.5
Escherichia coli	-	30±0.5	12±1.0	14±1.0	14±1.0	08±0.5	09±1.0	09±1.0	17±0.5	20±0.5	20±1.0	14±0.5	18±1.0	18±0.5
Klebsiella pneumoniae	-	26±0.5	-	-	-	-	-	-	16±0.5	18±0.5	18±1.0	08±1.0	08±1.0	08±1.0
Pseudomonas fluorescens	-	29±0.5	10±0.8	11±0.5	11±0.5	10±0.5	12±0.5	14±1.0	12±1.0	13±0.5	14±1.0	10±1.0	16±0.5	16±1.0.
Streptococcus pyogenes	-	30±0.5	12±0.5	13±0.5	13±0.5	10±0.5	12±0.8	13±1.0	08±10	08±1.0	10±1.0	11±1.0	14±0.5	14±1.0

Values are expressed as mean ± standard deviation of triplicates; NC - Negative control (DMSO); PC - Positive control (Streptomycin 30µg)

Table 3. Antifungal activity of different solvents like aqueous, methanol, ethanol and diethyl ether extracts of leaf of *M. quadrifolia*

Name of fungal species	NC	PC	Diameter of zone of inhibition (mm)											
			Aqueous extract			Methanol extract			Ethanol extract			Diethyl ether extract		
			50µl (2.5mg)	75µl (3.75mg)	100µl (5mg)	50µl (2.5 mg)	75µl (3.75 mg)	100µl (5mg)	50µl (2.5mg)	75µl (3.75mg)	100µl (5mg)	50µl (2.5mg)	75µl (3.75mg)	100µl (5mg)
Aspergillus niger	-	15±0.5	-	-	-	-	-	09±1.0	07±1.0	07±1.0	08±1.0	-	10±0.5	12±0.6
Aspergillus flavus	-	10±0.5	-	-	-	-	-	-	-	-	-	07±1.0	10±0.5	13±0.8
Aspergillus terreus	-	15±0.5	-	-	-	-	-	08±1.0	07±1.0	07±1.0	08±1.0	10±0.5	12±1.0	13±0.5
Trichoderma viride	-	16±0.5	-	-	-	-	-	08±1.0	-	-	-	09±1.0	09±1.0	11±0.5
Fusarium solani	-	16±0.5	-	-	-	-	-	07±1.0	-	-	-	11±0.5	11±0.5	12±0.5

Values are expressed as mean ± standard deviation of triplicates; NC - Negative control (DMSO); PC - Positive control (Amphotericin-B 50µg)

Table 4. Antifungal activity of different solvents like aqueous, methanol, ethanol and diethyl ether extracts of stem of *M. quadrifolia*

Name of fungal species	NC	PC	Diameter of zone of inhibition (mm)											
			Aqueous extract			Methanol extract			Ethanol extract			Diethyl ether extract		
			50µl (2.5mg)	75µl (3.75mg)	100µl (5mg)	50µl (2.5mg)	75µl (3.75mg)	100µl (5mg)	50µl (2.5mg)	75 µl (3.75mg)	100µl (5mg)	50µl (2.5mg)	75µl (3.75mg)	100µl (5mg)
Aspergillus niger	-	15±0.5	10±1.0	14±0.8	15±0.6	-	-	-	-	-	08±1.0	08±1.0	13±1.0	15±1.0
Aspergillus flavus	-	10±0.5	08±1.0	13±0.5	13±0.5	-	-	07±1.0	-	-	-	10±1.0	14±1.0	14±0.5
Aspergillus terreus	-	15±0.5	09±1.0	14±0.5	14±0.8	-	-	07±1.0	-	11±0.5	12±1.0	10±1.0	12±1.0	14±0.8
Trichoderma viride	-	16±0.5	10±1.0	13±0.5	14±1.0	-	-	10±1.0	09±1.0	10±0.5	11±1.0	12±0.5	15±0.5	15±0.6
Fusarium solani	-	16±0.5	11±1.0	13±1.0	13±0.5	-	-	08±1.0	08±1.0	12±0.5	12±0.5	10±0.5	13±0.5	14±1.0

Values are expressed as mean ± standard deviation of triplicates; NC - Negative control (DMSO); PC - Positive control (Amphotericin-B 50µg)

4. DISCUSSION

Presently there is an increasing interest worldwide on herbal medicines accompanied by increased laboratory investigation and pharmacological properties of the bioactive ingredients and their ability to treat various diseases [26,27]. Antibiotic resistance is increasing worldwide in both outpatients as well as hospitalized patients. It varies according to geographic locations and is directly proportional to the use and misuse of antibiotics. Resistance can now be demonstrated against all available classes of antibiotics [28].

Bacteria cause serious infection in humans as well as other animals. The rapid spread of bacteria expressing multidrug resistance (MDR) has necessitated the discovery of new antibacterials and resistance – modifying agents. There are many studies suggested that the necessity of developing alternative antimicrobial drugs [29,30]. Antimicrobials from plant source would be an excellent choice due to no side effects. A number of infectious agents are becoming more resistant to commercial antimicrobial compounds. So, there is a needful to develop new drugs, which requires varied strategies among them the secondary metabolites produced by medicinal plants are more important. According to World Health Organization (WHO) medicinal plants would be the best source to obtain a variety of drugs in developed countries.

Plants are used as medicine in many countries and are the source of potent and powerful drugs. Plants are the important source of potentially useful structures for the development of new chemotherapeutic agents. The first step towards this goal is the *In vitro* antibacterial activity assay. Medicinal plants represent a rich source of antimicrobial agents and many reports are available on antiviral, antibacterial and antifungal properties of plants [31,32].

Due to the extensive use of antibiotics the spread of multi resistant bacterial strains are one of the most worrying threats to public health [33]. Compared with Gram-positive bacteria, Gram-negative bacteria are more resistant against antibiotics, because of their impenetrable wall [34]. In the present study *M. quadrifolia* leaf extracts have showed effective inhibition against gram negative bacteria such as *Klebsiella pneumonia, Escherichia coli* and *Pseudomonas fluorescens* when compared to gram positive bacteria like *Bacillus subtilis* and *Streptococcus pyogenes*. These results are in accordance with the previous results of Ripa et al. [22], in that *M. quadrifolia* revealed profound antibacterial, cytotoxic and antioxidant effects. In this study, the antibacterial activity clearly showed that the leaf extract of *M. quadrifolia* was specific in action against the growth of pathogenic bacteria. Similar types of results like antibacterial activities of aqueous and methanol extracts of some medicinal plants were reported [35].

The antimicrobial properties of plants have been investigated by a number of researchers [36, 37]. Numerous investigations have proved that medicinal plants which exhibit various antimicrobial activities [38,39]. The use of plant extracts and phytochemicals, both with known antimicrobial properties, are of great significance to therapeutic treatments [40]. Extracts of plants were used for the treatment of various diseases caused by microorganisms. Mostly the pharmacological activity of medicinal plants resides in its secondary metabolites which provide clues to synthesize new antimicrobial and antifungal drugs [41].

Fungal diseases represent a critical problem to health and they are one of the main causes of morbidity and mortality worldwide [42]. Human infections, particularly those involving the skin and mucosal surfaces constitute a serious problem especially in tropical and subtropical

developing countries [43]. Infectious diseases accounts for high proportion of health problems in the developing countries. Microorganisms have developed resistance to many antibiotics and as a result, immense clinical problem in the treatment of infectious diseases has been created [44,45]. The present study revealed that profound antifungal activities of diethyl ether leaf and stem extracts of *M. quadrifolia* against fungal organisms.

Aqueous and diethyl ether extracts of stem of *M. quadrifolia* also showed potent antifungal activity against all tested fungal organisms. So it may also be useful in the treatment of fungal diseases caused by tested fungal organisms in this study. This study is also accordance with the antifungal activities of other medicinal plants [46,47]. *In vitro* antifungal activities of *Allium cepa* and *Allium sativum* against some pathogenic yeasts and dermatophytes were reported [48]. Antifungal activities of nine traditional Mexican medicinal plants [49] and traditional Indian medicinal plants [50] were reported and these results are accordance with the present study. So, this study reveals that antifungal effect of leaf and stem of *M. quadrifolia* against five different human pathogenic fungi including *Fusarium solani* and *Aspergillus species*. But the *Fusarium solani* and *Aspergillus* species are also plant pathogens [51] which infect the plants and produce diseases. So, we can use *M. quadrifolia* extracts as biopesticides in agriculture to protect the plants from fungal diseases.

In vitro antifungal activity of medicinal plants like of *Tribulus terrestris* L. against *Candida albicans*, *C. glabrata*, *C. parapsilosis*, *C. tropicalis* and *Cryptococcus neoformans* were reported [52]. Similar results were observed in the present study using *M. quadrifolia* extracts against different fungal organisms. The biological activity of the plant material depends on plant part, geographical source, harvest time, drying method and storage conditions. Therapeutic uses of medicinal plant are varied depends upon their parts like root, bark, stem, leaves and seeds [53]. In this study, we also observed different range of antimicrobial activities for leaf and stem of *M. quadrifolia.* Plants have the ability to synthesize secondary metabolites such as phenolics, nitrogen compounds, vitamins, terpenoids and some other endogenous metabolites. These substances serve as plant defense mechanism against predation by microbes, insects and herbivores [54]. In our previous study, there are many secondary metabolites were determined in leaf and stem extracts of *M. quadrifolia* by GC –MS analysis including the compounds with antimicrobial properties (data not shown). So, the antibacterial and antifungal activities of leaf and stem extracts of *M. quadrifolia* may be due to the presence of secondary metabolites with antimicrobial properties.

5. CONCLUSION

The present study clearly indicates that the *M. quadrifolia* had a profound antimicrobial activity and it may be useful in the treatment of various infectious diseases including skin transmitted infections. These results lend credence to the folkloric medicine, if this plant is used in treating microbial infection. In conclusion, the *M. quadrifolia* will be used to identify the new potent antimicrobial agents in future and it will also be useful to treat infectious diseases caused by microorganisms.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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