Original Article

In vitro Screening of Antibacterial and Antifungal Activity of *Marsilea quadrifolia* (Marsileaceae) Linn. Extract

T.G. Gini* and G. Jeya Jothi

Department of Plant Biology and Biotechnology, Loyola College, Chennai–600 034, Tamilnadu, India

Address for Correspondence

Department of Plant Biology and Biotechnology, Loyola College, Chennai–600 034, Tamilnadu, India.
E-mail: ginigeorgehc@gmail.com

ABSTRACT

Objectives: The purpose of the present research was to explore the antibacterial and antifungal activity of hexane, ethyl acetate, ethanol and methanol extracts of *Marsilea quadrifolia* against 31 species of bacteria and 7 species of fungi.
Methods: Antibacterial activity was assessed by Kirby-Bauer disc diffusion method. The concentrations of plant extracts were 1.25, 2.5 and 5 mg/disc. Streptomycin (10µg/disc) was used as standard antibiotic. The antifungal activity was assayed by minimum inhibitory concentration (MIC) by broth micro dilution method. The extracts were made in the concentration range of 0.010 to 0.0001 g/ml. Fluconazole was used as standard reference.
Results: Antibacterial assay revealed that the ethyl acetate extract (5 mg/disc) was effective against most of the bacterial strains, while the hexane, ethanol and methanol extracts were weakly effective. The diameter of inhibition zones was found to be ranged between 6-13 mm. Antifungal activity test showed that hexane extract (0.002 g/ml) was effective against *Aspergillus flavus* and *Candida albicans*. Ethyl acetate extract (0.002 g/ml) was effective against *Aspergillus flavus* and *Trichophyton rubrum*. Ethanol extract (0.005 g/ml and 0.002 g/ml) was effective against *Trichophyton mentagrophytes* and *Aspergillus flavus* respectively. Methanol extract was not effective against any of the fungal strains. The MIC ranged between 0.002-0.005 g/ml.
Conclusions: The results indicated that *Marsilea quadrifolia* extracts has antimicrobial activity and could be source of alternative antimicrobial drugs for treatment of diseases caused by the pathogens tested in this study.

Keywords: Antimicrobial drugs, *Marsilea quadrifolia*, Phytochemicals, Inoculum, Subculture.

INTRODUCTION

The continuous evolution of drug resistance in microorganisms to presently available antibiotics has demanded the search for alternate antibacterial compounds[1]. Efforts in this regard have focused on plants because of their historical use and the fact that a good portion of the world's population, particularly in developing nations, depend on plants for the cure of infectious and non infectious diseases[2-4]. Most of the medicinal plants are regarded as potential antimicrobial crude drugs as well as a source for novel compounds with antimicrobial activity, with possibly new modes of action. This prediction that some naturally existing plant compounds can kill antibiotic resistant strains of bacteria such as *Bacillus cereus*, *Escherichia coli*, *Micrococcus luteus* and *Staphylococcus aureus* has been confirmed, for example, by Friedman *et al*.[5] Pharmacological activity of most of the medicinal plants rests in the phyto-components present in them. These natural substances render clues to produce new structural types of antibacterial and antifungal agents which are comparatively safe to humankind[6]. There is account of number of studies performed on various plant extracts for antimicrobial activity and the discovery of new antimicrobial components[7]. Plant extracts represent a continuous effort to discover new antimicrobial compounds against several human pathogens[8].

Marsilea quadrifolia (*M. quadrifolia*) is an aquatic fern which anchors itself to the muddy bottoms of quiet, shallow lakes and streams. Commonly called as Four leaf clover, European water clover in English; Aalaikkeerai in Tamil; Neraral in Malayalam; Sunishanna, Chatuspatri in Sanskrit; Caupatiya, Sunsuniya in Hindi and is in use for more than 3000 years as part of food[9]. The plant produces roots both at the nodes and internodes of the rhizome. The slender petioles are usually glabrous (sometimes pubescent) and 5.5-17 cm (2-6 in.) long. They can occasionally reach 30 cm (1 ft.) if the plant is rooted deeply. The plant is anti-inflammatory, diuretic, depurative and refrigerant[10]. It is useful in psychopathy, leprosy, haemorrhoids, fever, diarrhea, insomnia and febrifuge[11]. Plants pacify vitiated pitta, cough, bronchitis, diabetes, psychiatric diseases, eye diseases and skin diseases. Previous studies have reported on antibacterial, psychopharmacological[9] and antioxidant activity of *M. quadrifolia*[12].

The phytocompounds that have been isolated from *M. quadrifolia* are marsilin (1 -triacontanol-cerotate), 3-hydoxy-triacontan-11 -one, hentriacontan-6-ol, methylamine, beta-sitosterol, marsileagenin A, flavonol-O-mono-and-diglycoside, C-glucoylflavones and C-glucosylxanthones[13]. The crude extract of *M. quadrifolia* caused prompt hypotensive response and is also found to be effective against electro convulsions[14]. *In vitro* cytotoxic activity of *M. quadrifolia* using MCF-7 cells of human breast cancer has been reported[15]. The objective of this investigation is to determine the presence and quantify the phytochemicals that are responsible for the antibacterial and antifungal activity in the whole plant extract of *M. quadrifolia*.

MATERIALS AND METHODS

Chemicals

The solvents hexane (H), ethyl acetate (EA), ethanol (ETOH) and methanol (MEOH) used for the extraction were of analytical grade (Merck). Dimethyl sulphoxide (DMSO) was from Fischer scientific. Mueller Hinton Agar (MHA), Mueller Hinton Broth (MHB), Sabouraud Dextrose Agar (SDA), Sabouraud Dextrose Broth (SDB), sterile discs, streptomycin

discs, 96 well microtitre plates were purchased from Hi media.

Collection and Identification of plant sample

The whole plant of *M. quadrifolia* was collected from Kalliyad (Thrissur, Kerala, India) in the month of April and May. The plant was identified by Dr. G. Jeya Jothi, Taxonomist and Assistant Professor, Department of Plant Biology and Biotechnology, Loyola College (Madras University), Chennai, India. Voucher specimen of the plant (*M. quadrifolia* - LCH 129) has been preserved in the Department of Plant Biology and Biotechnology, Loyola College for further reference.

Processing of plant materials

The plant materials were washed thoroughly under running tap water and shade dried for three weeks at room temperature. The dried plant materials were ground separately into a fine powder using an electric blender and were stored in airtight containers until use.

Preparation of plant extracts

Four different solvents namely hexane, ethyl acetate, ethanol and methanol were used for the sequential extraction started from low polarity to high polarity. Plant sample (50gm) was mixed with 300 ml of solvent and was placed on an orbital shaker at 120 rpm for 72 h at room temperature. The extraction process was carried out in triplicates with each solvent. The extracts were filtered through Whatman No.1 filter paper and concentrated at reduced pressure using a rotary vacuum evaporator. The dried crude extracts were kept in glass vials and stored in the refrigerator at 4 °C until use.

Test pathogens

The bacterial and fungal strains for the antimicrobial susceptibility testing were obtained from Microbial Type Culture Collection and Gene Bank (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh, India and Department of Microbiology, Christian Medical College, Vellore, Tamilnadu, India and National Chemical Laboratory (NCL), Pune, India. The bacterial strains were *Escherichia coli* (ATCC-25922), *Eubacterium lentum* (ATCC-43055), *Staphylococcus aureus* subsp. *aureus* (ATCC-25923), Methicillin sensitive *Staphylococcus aureus* (ATCC-29213), Methicillin Resistant *Staphylococcus aureus* (ATCC BAA-1761), *Klebsiella pneumoniae* subsp. *pneumoniae* (ATCC-700603), *Klebsiella pneumoniae* (ATCC BAA-1705), *Klebsiella pneumoniae* (ATCC BAA-1706), *Enterococcus faecalis* (ATCC-29212), *Acinetobacter baumannii* (ATCC-17978), *Vibrio fischeri* (ATCC-7744), *Yersinia enterocolitica* (MTCC-840), *Enterobacter aerogens* (MTCC-111), *Erwinia amylovora* (MTCC-2760), *Proteus vulgaris* (MTCC-1771), *Bacillus subtilis* (MTCC-441), *Staphylococcus epidermidis* (MTCC-3615), *Salmonella enterica typhimurium* (MTCC-1251), *Salmonella paratyphi* (MTCC-3220) *Staphylococcus aureus* (MTCC-96), *Micrococcus luteus* (MTCC-106), *Vibrio parahaemolyticus* (MTCC-451), *Enterococcus durans* (MTCC-3031), *Pseudomonas fluorescens* (MTCC-2421), *Trichoderma sp.* (MTCC-3471), *Bacillus subtilis* (MTCC-1305), *Escherichia coli* (MTCC-1721), *Enterococcus faecalis* (MTCC-2729), *Acinetobacter baumannii* Carbapenem Resistant (ICMR-19), *Escherichia coli* Cipro Resistant (ICMR-24) and *Enterococcus faecalis* (NCL–5025). The fungal strains were *Candida albicans* (MTCC-227), *Aspergillus niger* (MTCC-281), *Aspergillus flavus* (MTCC-277), *Trichophyton rubrum* (MTCC-296), *Trichophyton mentagrophytes* (MTCC-

8476), *Curvularia lunata* (MTCC-2030), *Botrytis cinerea* (MTCC-359).

Culture maintenance and inoculum preparation

The bacterial and fungal strains were preserved at 4^0C on Mueller Hinton agar and Sabouraud Dextrose agar slants respectively. The pure stock cultures were subsequently subcultured into newly prepared Mueller Hinton agar and Sabouraud Dextrose slants in the laboratory. Inoculums of bacteria were prepared by suspending a loop full of bacterial cultures into 5 ml of Mueller Hinton broth and were incubated at 37^0C for 24 h on a rotary shaker. Inoculums of fungi were prepared by collecting fungal spores from freshly subcultured fungal strains and suspending into 5 ml of sterile distilled water under aseptic conditions prior to antifungal assay.

Antibacterial susceptibility testing

In vitro antibacterial susceptibility testing was determined by Kirby-Bauer disc diffusion method[16]. The medium was prepared by dissolving 38 gm of the commercially available Mueller Hinton agar medium in 1000 ml of distilled water. The dissolved medium was autoclaved at 15 lbs pressure at 121°C for 15 min. The autoclaved medium was mixed well and poured into 90 mm disposable sterile petriplates (25-30 ml/plate) while still molten and the plates were allowed to solidify under sterile condition at room temperature. After solidification and drying, the plates were seeded with overnight grown culture of each pathogen by swabbing evenly on to the surface of the medium with a sterile cotton swab. The inoculums were allowed to dry for 5 min. The plant extracts 1.25, 2.5 and 5 mg/disc were prepared by dissolving in Dimethyl sulphoxide. The sterile discs were then infused with the extracts and were positioned on the surface of the medium with sterile forceps and gently pressed down to ensure contact with the agar. Streptomycin (10 µg/disc) was used as standard antibiotic. Then the plates were inverted and incubated at 37^0C for 24 h to allow perfusion of drugs being tested. The next day each plate was examined and the antibacterial activities of discs were determined by measuring the zone of inhibition expressed in millimeters. The results were interpreted by the presence or absence of zone of inhibition.

Antifungal activity testing

The antifungal activity was determined by minimum inhibitory concentration (MIC) by broth micro dilution method approved by the National Committee for Clinical Laboratory Standards (NCCLS)[17] with slight modification. The 96-well microtitre plates were prepared by dispensing into each well 100 µl of Sabouraud Dextrose Broth (SDA). The plant extracts were dissolved in Dimethyl Sulphoxide (DMSO). The initial concentration of the extracts was 10 mg/ml. The initial test concentration was serially diluted two-fold. The extracts were made in the concentration range of 0.010 to 0.0001 g/ml. The last well contained broth and inoculum without extract which was kept as negative control. Fluconazole was used as positive control. The fungal inoculum was prepared by collecting spores from the freshly subcultured fungal strains into sterile distilled water. 20 µl of the fungal inoculum was added to each of the well. The final concentration in each well was 120 µl. The microtitre plates were capped and sealed well. It was then incubated at 28^0C for 72 h. Based on the visible turbidity, which was representative of a growth of test organism, the MIC was examined. The minimum concentration at which the growth was suppressed as compared to the control was confirmed as MIC.

Determination of the minimum fungicidal concentration (MFC)

Aliquots (2 µl) of the MIC wells were transferred into Sabouraud Dextrose Agar (SDA) plates without the drug. The plates were incubated at 28°C for 48 h. The Minimum Fungicidal Concentration (MFC) was determined as the lowest concentration of the crude extract that was capable of preventing growth of fungi.

RESULTS AND DISCUSSION

Antibacterial activity

Antibacterial susceptibility assay revealed that the highest zone of inhibition was exhibited by ethyl acetate extract when compared to hexane, ethanol and methanol extracts of *M. quadrifoila* against number of bacteria. The inhibition zone ranged from 6 to 13mm in diameter. Ethyl acetate extract (5 mg/disc) showed the zone of inhibition against *E. coli*- Cipro Resistant (13 mm), *E. amylovora* (12 mm), *Y. enterocolitica, S. aureus, K. pneumoniae* and *E. lentum* (11 mm each), *P. vulgaris, S. paratyphi* and *P. fluorescence* (10 mm each), *E. coli, S. aureus* Methicillin sensitive, *E. faecalis*, *S. enterica typhimurium* and *V. fischeri* (9 mm each). Ethyl acetate extract (2.50 mg/disc) showed the zone of inhibition against *E. coli*- Cipro Resistant (10 mm), *Y. enterocolitica*, *S. aureus*, *E. amylovora*, *K. pneumoniae* and *P. fluorescence* (9 mm each). Ethyl acetate extract (1.25 mg/disc) showed the zone of inhibition against *P. fluorescence* (10 mm) and *M. luteus* and *E. coli*- Cipro Resistant (9 mm each). The inhibition zone against *E. coli* (MTCC–1721) was found to be 10 mm in all the three concentrations. The inhibition zone exhibited by ethyl acetate extract against other bacterial strains was between 6–8 mm. The maximum inhibition zone exhibited by hexane extract in 5 mg/disc concentration was against *E. amylovora* and *E. coli* (10 mm) followed by 9 mm against *M. luteus*. The hexane extract (2.50 mg/disc) was active against *E. coli* with inhibition zone as 10 mm and *E. amylovora* as 9 mm. The hexane extract concentration 1.25 mg/disc showed 9 mm as the largest inhibition zone against *K. pneumoniae*. The hexane extract was more ineffective against rest of the bacterial strains.

The highest zone of inhibition for ethanol extract was 9 mm. The ethanol extract was effective against *E. aerogens* in all three concentrations with the similar zone of inhibition (9 mm). Ethanol extract also showed 9 mm zone of inhibition in different concentrations against *B. subtilis* (5 mg/disc), *S. aureus* subsp. *aureus* (2.50 mg/disc), *P. vulgaris* and *P. fluorescence* (1.25mg/disc) respectively. The inhibition zone was between 6 and 8 mm against the other bacterial strains. The methanol extract exhibited the highest inhibition zone against *B. subtilis* (10 mm) followed by *E. lentum* (9 mm) in 5 mg/disc concentration. The zone of inhibition was 9 mm against *S. aureus* subsp. *aureus* and *M. luteus* in 2.50 mg/disc concentration. The inhibition zone ranged between 6 and 8 mm in 1.25mg/disc concentration of methanol extract.

Antifungal activity

The antifungal activity was measured by minimum inhibitory concentration (MIC) by broth micro dilution method. The antifungal assay showed that *M. quadrifolia* has antifungal property against *A. flavus, T. mentagrophytes*, *T. rubrum* and *C. albicans*. *M. quadrifolia* extracts were not effective against *A. niger*, *B. cinerea* and *C. lunata*. The hexane extract (0.002 g/ml) was effective against *A. flavus* and *C. albicans*. Ethyl acetate extract showed antifungal property against *A. flavus* and *T. rubrum* in concentration 0.002 g/ml. Ethanol extract inhibited the growth of *T. mentagrophytes* and *A. flavus* in the concentration 0.005 g/ml and 0.002 g/ml respectively. The methanol

extract was not effective against any of the tested fungi. These findings revealed that *M. quadrifolia* was comparatively more effective against *A. flavus* in comparisons to *T. mentagrophytes*, *T. rubrum* and *C. albicans*.

Determination of minimum fungicidal concentration (MFC)

Minimum Fungicidal Concentration was determined by transferring aliquots (2 µl) of the MIC wells into Sabouraud Dextrose Agar (SDA) plates without the drug. The Minimum Fungicidal Concentration confirmed that *M. quadrifolia* extracts prevented the growth of *A. flavus* (0.002 g/ml), *T. mentagrophytes* (0.005 g/ml), *T. rubrum* (0.002 g/ml) and *C. albicans* (0.002 g/ml). The present antifungal agents commonly used have toxicity problems on the host organism as well as both the pathogen and the hosts have eukaryotic set up of cellular organization[18].

The present findings showed that *M. quadrifolia* has both antibacterial and antifungal properties against the tested pathogens. However the plant did not exhibit a strong antimicrobial activity. The negative results do not mean that the plant is less active and lacked bioactive compounds. Bioactive compounds may be present in insufficient quantities in the crude extracts to show its effect with the dose levels employed[19]. Lack of activity can thus only be proven by using large doses[20]. Alternatively, if the active principle is present in high quantities, there could be other constituents wielding antagonistic effects or nullifying the positive effects of the bioactive agents[21].

Antibacterial assay revealed that the ethyl acetate extract was comparatively more effective against most of the bacterial strains, in comparisons to hexane, ethanol and methanol extracts. Ethyl acetate extract was more effective against *E. coli*- Cipro Resistant, *E. amylovora*, *Y. enterocolitica, S. aureus, K. pneumoniae*, *E. lentum*, *P. vulgaris, S. paratyphi*, *P. fluorescence*, *E. coli, S. aureus* Methicillin sensitive, *E. faecalis*, *S. enterica typhimurium*, *V. fischeri* and *M. luteus*. Ethyl acetate extract was weekly effective against the rest of the test pathogens. The hexane extract was effective against *E. amylovora*, *E. coli* (MTCC–1721), *M. luteus* and *K. pneumoniae* (ATCC BAA- 1706). The hexane extract was completely ineffective against *E. coli* (ATCC-25922), *B. subtilis*, *V. parahaemolyticus*, *E. faecalis*, *A. baumannii*, *K. pneumoniae* (ATCC BAA- 1705), *V. fischeri*, *P. fluorescence*, *S. epidermidis* and *Trichoderma sp.*

The ethanol extract was active against *E. aerogens*, *B. subtilis*, *S. aureus* subsp. *aureus*, *P. vulgaris*, *E. lentum*, *M. luteus* and *P. fluorescence*. Ethanol extract was completely ineffective against *E. coli* (ATCC-25922), *S. aureus* (Methicillin sensitive), *V. parahaemolyticus*, *S. epidermidis*, *A. baumannii*- Carbapenem Resistant, *K. pneumoniae* (ATCC BAA-1706), *E. lentum*, *S. enterica typhimurium* and *V. fischeri*. The methanol extract was effective against *B. subtilis*, *E. lentum*, *S. aureus* subsp. *aureus* and *M. luteus*. Methanol extract was totally ineffective against *E. coli* (ATCC-25922), *A. baumannii* -Carbapenem Resistant, *E. faecalis* (MTCC-2729), *K. pneumoniae* (ATCC BAA-1706), *K. pneumonia* (ATCC- 700603), *S. enterica typhimurium* and *P. fluorescence*.

Antifungal activity test showed that *M. quadrifolia* extracts were active against *A. flavus, T. mentagrophytes*, *T. rubrum* and *C. albicans* and were not effective against *A. niger*, *B. cinerea* and *C. lunata*. The inhibition concentration ranged between 0.002 g/ml and 0.005 g/ml. Hexane, ethyl acetate and ethanol extracts were active against *A. flavus* in the same concentration range (0.002 g/ml). The concentration of the

extracts affected the rate of inhibition of growth of pathogens. Antibacterial and antifungal assays revealed that higher the concentration of the crude extracts higher the rate of inhibition. The ethyl acetate extract of *M. quadrifolia* showed the most remarkable activity. This plant could be further subjugated to isolation of the therapeutic antimicrobial drugs and carry out further pharmacological evaluation.

CONCLUSION

The current findings can be used in further exploration of this medicinal plant in isolation of the antimicrobial agents responsible for these properties. This investigation has revealed that this plant specimen studied has both antibacterial and antifungal activity on the tested human pathogens in this research. The ethyl acetate extract of *M. quadrifolia* exhibited the most remarkable activity against bacteria when compared to hexane, ethyl acetate and ethanol extracts. The hexane, ethyl acetate and ethanol extracts of *M. quadrifolia* were effective against fungi, while methanol extract was totally ineffective. This is an indication that this plant could be of high medicinal value, which could be exploited to be used in the formation of alternative antimicrobial drugs against number of human diseases. The phytochemical characterization of the extracts, the identification of responsible bioactive compounds and quality standards are indispensable.

Conflict of interest

We declare that we have no conflict of interest.

ACKNOWLEDGEMENT

The first author (T.G.G.) thanks profoundly University Grants Commission (MANF Scheme) for funding this project. We express our deep sense of gratitude to Dr. G. Joseph Anthony Samy, S.J., Principal, Loyola College, for his constant encouragement and steering and we are thankful to Professor Antoine Label, Head of the Department of Plant Biology and Biotechnology, Loyola College, Chennai, for his valuable support.

REFERENCES

1. Akinnibosun FI, Akinnibosun HA, Ibeh IN, Osaghae F. Antibacterial activity of *Phyllanthus amarus Schum. and Thonn.* on five vegetative organisms. *Plant Archives*. 2008; 8(2): 563-568.
2. Martinez MJ, Betamcourt J, Alonso-Gonzalez N, Jauregai A. Screening of some Cuban medicinal plants for antimicrobial activity. *Journal of Ethnopharmacology*. 1996; 52:171-174.
3. Jadeja DJ, Parekh, Chanda S. Efficiency of aqueous and methanol extracts of some medicinal plants for potential antibacterial activity. *Turkish Journal of Biology*. 2005; 29:203-210.
4. Aibinu I. Medicinal plants as antimicrobials. In: T. Odugbemi (Ed.). Outlines and pictures of medicinal plants from Nigeria. University of Lagos Press, Akoka, Yaba, Lagos State, Nigeria. 2006.
5. Friedman DB, Stauff DL, Pishchany G, Whitwell CW, Torres VJ *et al*. *Staphylococcus aureus* Redirects Central Metabolism to Increase Iron Availability. *Journals plos pathogens*. 2006; 2(8): e87.
6. Kalimuthu KS, Vijayakumar, Senthilkumar R. Antimicrobial activity of the Biodiesel plant *Jatropha curcas* L. *International Journal of Pharma and Bio Sciences*. 2010; 1(3): 1-5.
7. Sharma S, Vijayvergia R, Singh T. Evaluation of antimicrobial efficacy of some medicinal plants. *Journal of chemical and Pharmaceutical Research*. 2010; 2(1): 121-124.
8. Ahmad N, Amir MK, Ayaz S, Ahmad S, Jan A, Ashraf JS, Zuhra FT. Antimicrobial Profile of the Selected Medicinal Plants.

International Journal of Chemical and Life Sciences. 2012; 1(2): 1039-1041.

9. Ayurvedic Medicinal plants, Toxicology centre.com retrieved October 10, 2013 from http://www.toxicologycentre.com/English/plants/Malayalam/neeraral.html.
10. Schofield JJ. "Discovering Wild plants, Alaska, Western Canada, the Northwest". Alaska Northwest Books, G.TE Discovery Publications, Inc. 22023 20th Ave. S.E. Bothell, WA. 98021, 1989.
11. Longman O. Indian medicinal plants, vol. 4, Orient Longman Pvt. Ltd., Chennai, India, 1997; pp. 5-9.
12. Reddy KS, Reddy CS, Ganapaty S. Psychopharmacological Studies of Hydro Alcoholic Extract of Whole Plant of *Marsilea quadrifolia*. *Journal of Scientific Research*. 2012; 4 (1), 279-285.
13. Farhana AR, Laizuman N, Mahmuda H, Md. Monirul Islam. Antibacterial, Cytotoxic and Antioxidant Activity of Crude Extract of *Marsilea quadrifolia*. *European Journal of Scientific Research*. 2009; 33 (1), pp.123-129.
14. Khare CP. Encyclopedia of Indian Medicinal Plants, Rational western theraphy, Ayurvedic and other Traditional Uses, Botany (Springer, London) 2004; p. 303.
15. Uma R, Pravin B. Invitro Cytotoxic Activity of *Marsilea quadrifolia Linn* of MCF-7 Cells of Human Breast Cancer, *International Research Journal of Medical Sciences*. 2013; 1(1), 10-13.
16. Kirby –Bauer Disk Diffusion Susceptibility Test Protocol, retrieved November 19, 2013 from http://www.microbelibrary.org/component/resource/laboratory-test/3189-kirby-bauer-disk-diffusion-susceptibility-test-protocol.
17. NCCLS. Reference method for broth dilution antifungal susceptibility testing of filamentous fungi. Approved standard M38-A. Wayne, Pa: National Committee for Clinical Laboratory Standards; 2002.
18. Krishnan S, Manavathu EK, Chandrasekar PH. *Aspergillus flavus*: An emerging non-*fumigatus Aspergillus* species of significance. *Mycoses*. 2009; 52:206–222.
19. Taylor JLS, Rabe T, McGraw LJ, Jager AK, van Staden J. Towards the scientific validation of traditional medicinal plants. *Plant Growth Regul*. 2001; 34: 23-37.
20. Farnsworth NR. Biological approaches to the screening and evaluation of natural products. In: Rasoanaivo P, Ratsimamanga-Urverg S (Eds) Biological Evaluation of Plants with Reference to the Malagasy Flora, Madagascar, 1993; pp. 35-43.
21. Jager AK, Hutchings A, van Staden J. Screening of Zulu medicinal plants for prostaglandin- synthesis inhibitors. *J. Ethnopharmacol*. 1996; 52:95-100.

Table 1. Antibacterial activity of Hexane and Ethyl acetate extracts of *Marsilea quadrifolia* Linn.

Code No.	Name of the Microorganisms	*Marsilea quadrifolia* (Zone of inhibition in mm)						Standard Antibiotic 10 µg/disc
		Hexane			Ethyl acetate			
		1.25 mg/disc	2.50 mg/disc	5.0 mg/disc	1.25 mg/disc	2.50 mg/disc	5.0 mg/disc	
ATCC -25922	*Escherichia coli*	-	-	-	8	8	9	15
MTCC- 1771	*Proteus vulgaris*	7	8	7	6	8	10	14
MTCC - 840	*Yersinia enterocolitica*	6	7	8	8	9	11	10
MTCC - 111	*Enterobacter aerogens*	-	-	7	-	-	7	15
ATCC- 29213	*Staphylococcus aureus* Methicillin sensitive	7	-	8	6	7	9	20
MTCC- 3220	*Salmonella paratyphi*	-	6	6	7	8	10	11
MTCC- 441	*Bacillus subtilis*	-	-	-	-	7	8	10
MTCC - 3471	*Trichoderma sp.*	-	-	-	-	-	7	11
MTCC- 451	*Vibrio parahaemolyticus*	-	-	-	-	7	8	20
NCL - 5025	*Enterococcus faecalis*	-	-	-	7	7	8	20
ATCC-25923	*Staphylococcus aureus* subsp. *aureus*	-	-	6	-	-	6	16
MTCC- 3615	*Staphylococcus epidermidis*	-	-	-	8	-	-	20
MTCC- 106	*Micrococcus luteus*	6	8	9	9	-	-	18
MTCC- 96	*Staphylococcus aureus*	7	8	7	8	9	11	10
MTCC -2760	*Erwinia amylovora*	8	9	10	8	9	12	24
ICMR -19	*Acinetobacter baumannii*- Carbapenem Resistant	6	7	-	6	7	8	9
MTCC- 1305	*Bacillus subtilis*	-	-	-	6	7	8	20
ATCC- 17978	*Acinetobacter baumannii*	-	-	-	-	7	8	18
MTCC- 1721	*Escherichia coli*	7	10	10	10	10	10	17
ATCC- 29212	*Enterococcus faecalis*	6	7	7	7	8	8	10
MTCC- 2729	*Enterococcus faecalis*	-	7	7	7	8	9	9
ATCC BAA-1705	*Klebsiella pneumoniae*	-	-	-	7	8	8	16
ATCC BAA-1706	*Klebsiella pneumoniae*	9	7	7	6	6	6	15

ATCC- 700603	*Klebsiella pneumoniae*	7	8	8	8	9	11	17
ATCC- 43055	*Eubacterium lentum*	6	6	7	7	8	11	18
MTCC- 1251	*Salmonella enterica typhimurium*	7	7	7	7	8	9	18
MTCC- 3031	*Enterococcus durans*	6	7	7	6	6	6	-
ICMR - 24	*Escherichia coli- Cipro Resistant*	6	7	8	9	10	13	11
ATCC BAA-1761	Methicillin Resistant *Staphylococcus aureus*	-	7	8	-	-	6	10
ATCC- 7744	*Vibrio fischeri*	-	-	-	7	8	9	9
MTCC- 2421	*Pseudomonas fluorescence*	-	-	-	10	9	10	13

- = no zone of inhibition

Table 2. Antibacterial activity of Ethanol and Methanol extracts of *Marsilea quadrifolia* Linn.

Code No.	Name of the Microorganisms	*Marsilea quadrifolia* (Zone of inhibition in mm)						Standard Antibiotic 10 µg/disc
		Ethanol			Methanol			
		1.25 mg/disc	2.50 mg/disc	5.0 mg/disc	1.25 mg/disc	2.50 mg/disc	5.0 mg/disc	
ATCC -25922	*Escherichia coli*	-	-	-	-	-	-	13
MTCC- 1771	*Proteus vulgaris*	9	7	-	7	6	6	10
MTCC – 840	*Yersinia enterocolitica*	-	6	6	-	6	6	8
MTCC – 111	*Enterobacter aerogens*	9	9	9	8	-	8	11
ATCC- 29213	*Staphylococcus aureus* (Methicillin sensitive)	-	-	-	-	6	7	18
MTCC- 3220	*Salmonella paratyphi*	6	6	7	7	7	7	10
MTCC- 441	*Bacillus subtilis*	-	-	8	7	7	7	10
ATCC - 3471	*Trichoderma sp.*	-	7	7	7	7	7	16
MTCC- 451	*Vibrio parahaemolyticus*	-	-	-	-	7	8	20
NCL - 5025	*Enterococcus faecalis*	6	8	8	-	-	7	25
ATCC-25923	*Staphylococcus aureus* subsp. *aureus*	7	9	8	6	9	8	14
MTCC- 3615	*Staphylococcus epidermidis*	-	-	-	-	6	7	14

MTCC- 106	*Micrococcus luteus*	6	6	7	8	9	8	15
MTCC- 96	*Staphylococcus aureus*	8	7	7	7	7	6	8
MTCC -2760	*Erwinia amylovora*	7	8	7	-	6	7	9
ICMR -19	*Acinetobacter baumannii*- Carbapenem Resistant	-	-	-	-	-	-	9
MTCC- 1305	*Bacillus subtilis*	7	8	9	7	8	10	22
ATCC- 17978	*Acinetobacter baumannii*	-	-	7	-	7	8	20
MTCC- 1721	*Escherichia coli*	7	-	6	7	-	8	17
ATCC- 29212	*Enterococcus faecalis*	7	-	-	-	7	-	8
MTCC- 2729	*Enterococcus faecalis*	-	8	-	-	-	-	8
ATCC BAA-1705	*Klebsiella pneumoniae*	6	-	-	6	8	6	15
ATCC BAA-1706	*Klebsiella pneumoniae*	-	-	-	-	-	-	16
ATCC- 700603	*Klebsiella pneumonia*	-	-	6	-	-	-	16
ATCC- 43055	*Eubacterium lentum*	-	-	-	7	8	9	12
MTCC- 1251	*Salmonella enterica typhimurium*	-	-	-	-	-	-	15
MTCC- 3031	*Enterococcus durans*	-	-	8	6	7	6	15
ICMR -24	*Escherichia coli- Cipro* Resistant	6	6	7	6	7	8	12
ATCC BAA-1761	Methicillin Resistant *Staphylococcus aureus*	7	8	7	-	-	7	11
ATCC- 7744	*Vibrio fischeri*	-	-	-	-	6	7	9
MTCC- 2421	*Pseudomonas fluorescence*	9	8	7	-	-	-	13

- = no zone of inhibition

 ISSN 2321 – 2748

Table 3. Minimum Fungicidal Concentration (MFCs) of *Marsilea quadrifolia* Linn. extracts against tested Fungi (g/ml)

Code No.	Test Microorganisms	Hexane	Ethyl acetate	Ethanol	Methanol	Fluconazole
MTCC- 227	*Trichophyton mentagrophytes*	-	-	0.005	-	0.005
MTCC- 281	*Aspergillus niger*	-	-	-	-	0.010
MTCC-277	*Aspergillus flavus*	0.002	0.002	0.002	-	0.010
MTCC- 296	*Botrytis cinerea*	-	-	-	-	0.002
MTCC- 8476	*Trichophyton rubrum*		0.002	-	-	0.005
MTCC-2030	*Curvularia lunata*	-	-	-	-	0.002
MTCC- 359	*Candida albicans*	0.002	-	-	-	0.002

 ISSN 2321 – 2748

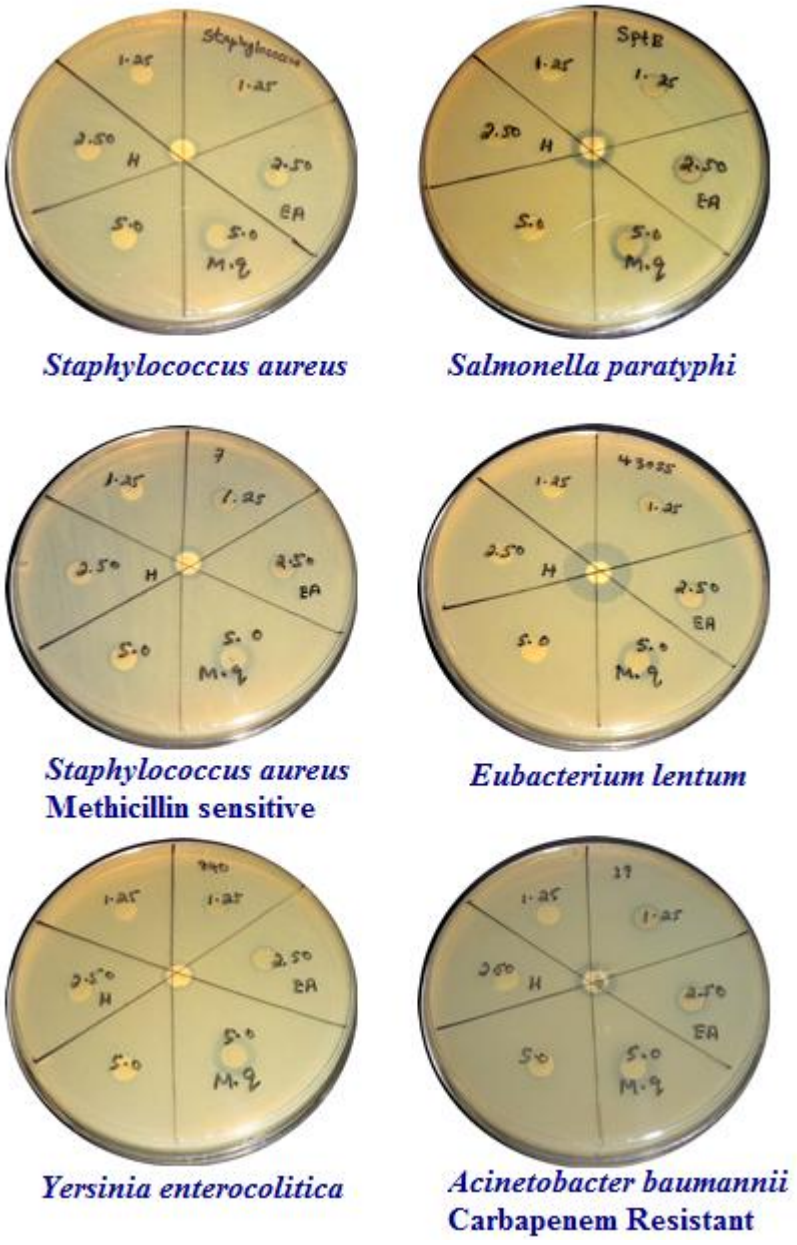

Figure 1. Zone of inhibition is shown by the hexane and ethyl acetate extracts of *Marsilea quadrifolia* against tested bacteria

 ISSN 2321 – 2748

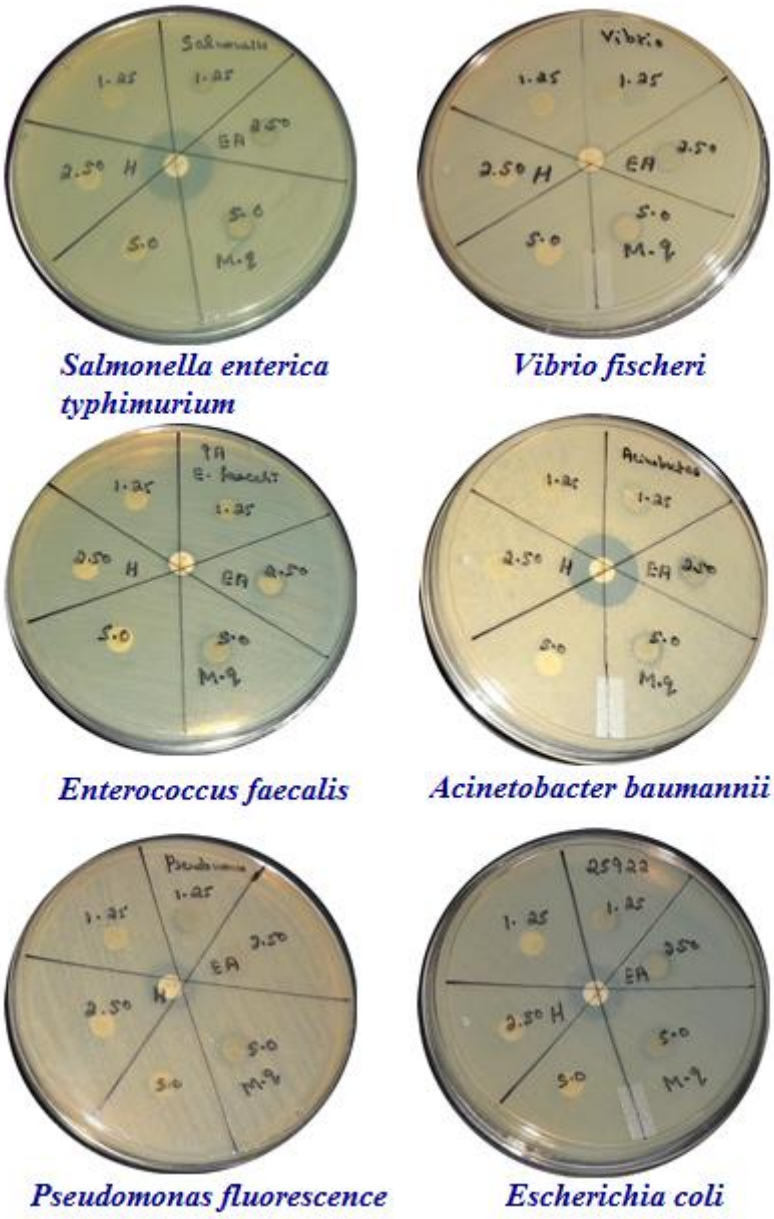

Figure 2. Hexane and ethyl acetate extracts of *Marsilea quadrifolia* showing zone of inhibition against tested bacteria

 ISSN 2321 – 2748

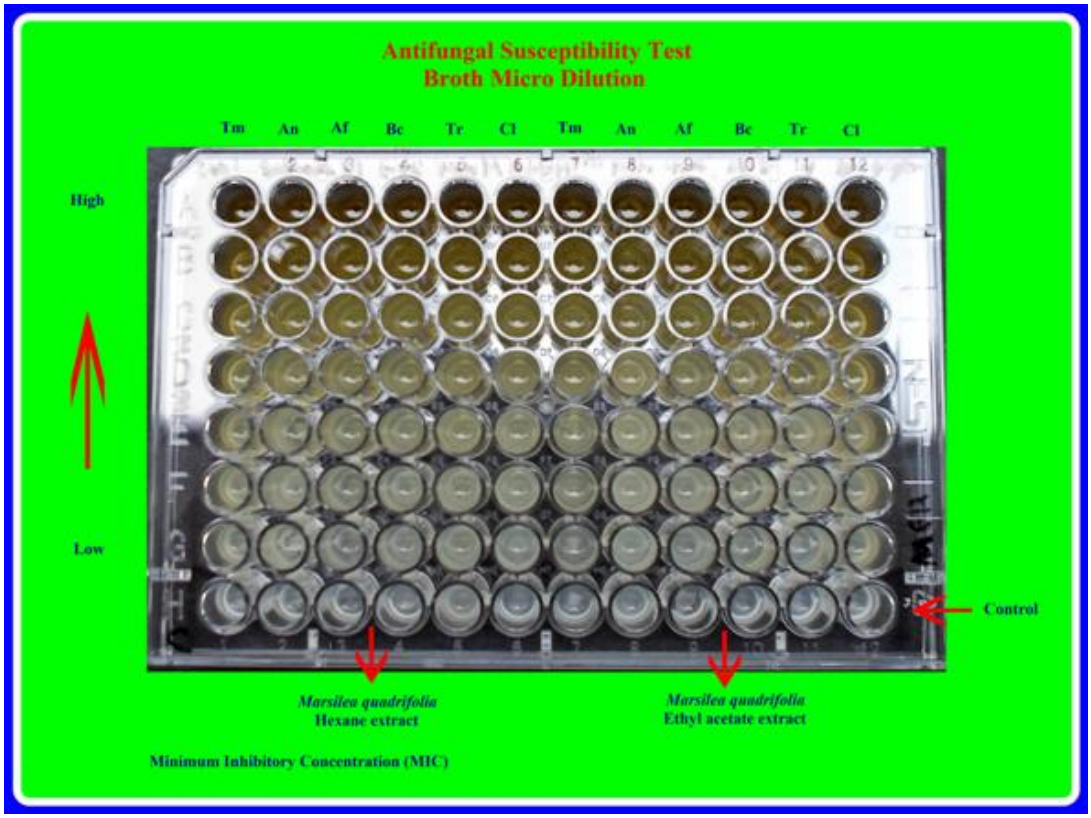

Figure 3. Minimum inhibitory concentration exhibited by hexane and ethyl acetate extracts of *Marsilea quadrifolia* against tested fungi

(Tm: *Trichophyotn mentagrophytes*, An: *Aspergillus niger*, Af: *Aspergillus flavus*, Bc: *Botrytis cinerea*, Tr: *Trichophyton rubrum*, Cl: *Curvularia lunata*)

 ISSN 2321 – 2748

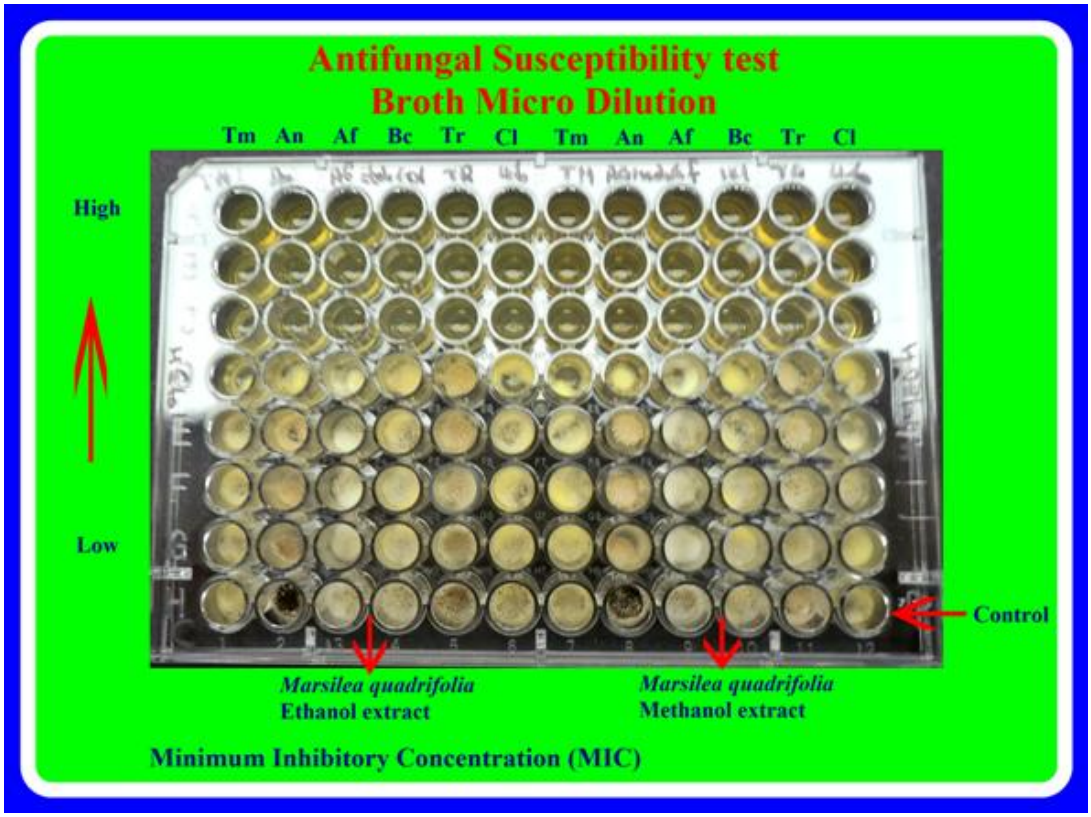

Figure 4. Minimum inhibitory concentration exhibited by ethanol and methanol extracts of *Marsilea quadrifolia* against tested fungi

(Tm: *Trichophyotn mentagrophytes*, An: *Aspergillus niger*, Af: *Aspergillus flavus*, Bc: *Botrytis cinerea*, Tr: *Trichophyton rubrum*, Cl: *Curvularia lunata*)

 ISSN 2321 – 2748

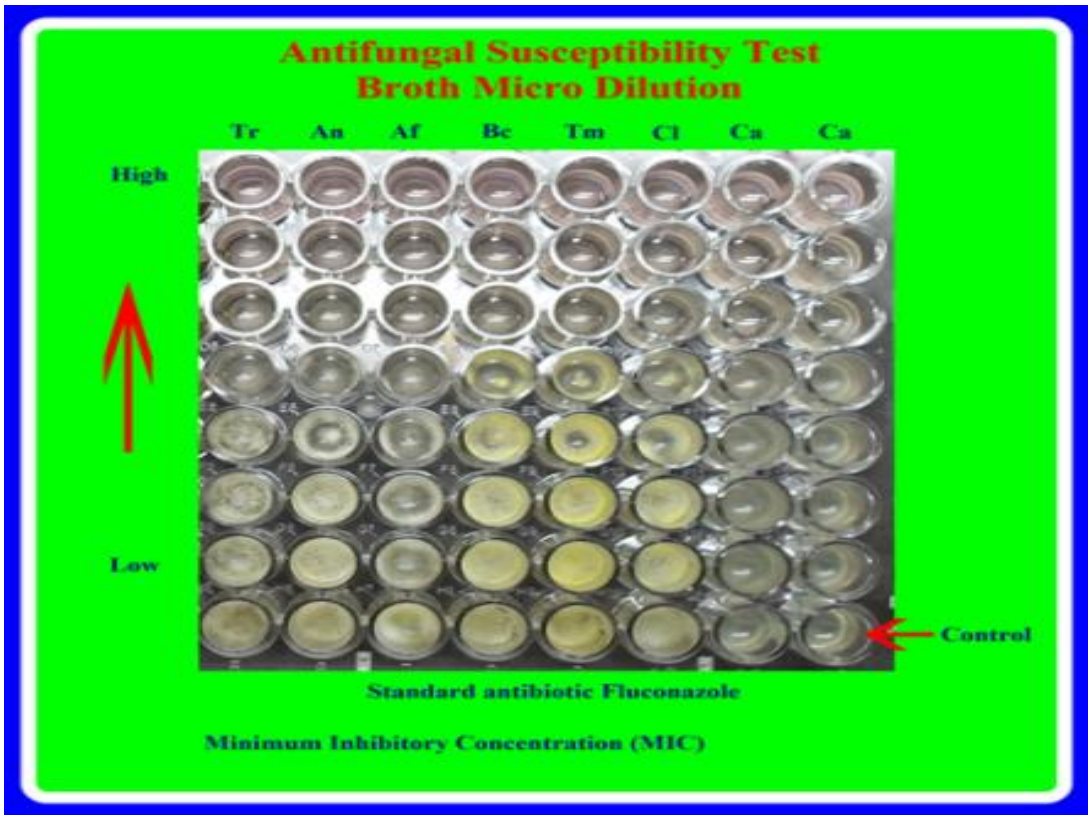

Figure 5. Minimum inhibitory concentration shown by the Standard antibiotic Fluconazole against tested fungi

(Tm: *Trichophyotn mentagrophytes*, An: *Aspergillus niger*, Af: *Aspergillus flavus*, Bc: *Botrytis cinerea*, Tr: *Trichophyton rubrum*, Cl: *Curvularia lunata*, Ca: *Candida albicans*)