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EVALUATION OF ANTIFUNGAL POTENTIAL OF *MARCHANTIA LINEARIS* LEHM & LINDENB. A BRYOPHYTE AGAINST FUNGAL PATHOGENS

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Marchantia linearis Lehm and Lindenb. a bryophyte was evaluated for its antifungal activity associated with its secondary metabolites. The thallus was successively extracted with various solvents by hot continuation extraction using soxhlet apparatus to detect the presence of different phytochemicals such as phenols, flavonoids, carbohydrates, glycoproteins, alkaloids, sterols and triterpenes. Phenols and flavonoids are present at significant levels in the ethanolic extract than water. Antifungal evaluation was carried on both ethanolic and water extracts by micro spectrophotometric assay. Minimal inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) were determined. Crude ethanolic exract of *Marchantia linearis* exerted significant fungicidal activity against *Candida albicans, Botrytis cinerea, Colletotrichum capsici, Fusarium oxysporum, Fusarium solani, Phytophthora capsici, Rhizoctonia solani* and *Sclerotinia sclerotiorum* at varied levels. The MIC values of the extracts were determined ranging between 125 to 2000 µg/ml. The MFC values of the extracts ranged between 500 to 2500 µg/ml. MICs and MFCs values obtained were quite comparable to values obtained with the fungicide fluconazole (125-1000 µg/ml).

Keywords : Antifungal activity; Ethanol; Inhibition; *Marchantia linearis;* Photyochemical.

Introduction

Ethnobotanical studies revealed that a wider range of Indian plants are being usd in the treatment of wounds and other disease in the traditional health care system of the country[1]. Plants therapeutic essence is its secondary metabolites, known as phytochemicals. These organic chemical substances are stored in matured cells of the various organs such as tannins, terpenoids, alkaloids, and flavanoids which possess potential *in vitro* antimicrobial properties. With increasing awareness of environmental issues, the trend nowadays in towards developing sustainable agricultural ecosystems. Some key components of sustainability include integrated crop management, organic agriculture and to pest management that reduce or eliminate the use of toxic pesticides. The development of safe and effective antimicrobial drugs has revolutionized medicine in the last forty years, so that morbidity and mortality from microbial diseases have been drastically reduced by modern chemotherapy. However, the current rates of resistance and cross-resistance development to all available classes of antibiotics agents has necessitated the search for new antimicrobial substances, with novel antimicrobial mechanisms. The increase in prevalence of multiple drug resistance has slowed down the development of new synthetic antimicrobial drugs, and has necessitated the search for natural antimicrobials from alternative sources. Recent progress to discover drugs from natural sources has resulted in compounds that are being developed to treat cancer, resistant bacteria, viruses and immunosuppressive disorders[2]. Phytochemicals from medicinal plants showing antimicrobial activities have the potential of filling this need, because their structures are different from those of the more studied microbial sources, and therefore their mode of action are also very likely to differ. There is growing interest in correlating the phytochemical constituents of a medicianl plant with its pharmacological activity[3]. Screening the active compounds from plants has lead to the discovery of new medicinal drugs which have efficient protection and teatment roles against various diseases[4]. From numerous studies, it is evident that these antimirobial agents from higher plants are plentiful and that there is a large amount of unexploited natural sources of antimicrobial compounds in lower plants. It is desirable to search for new types of biopesticides from lower plant groups.

Table 1. Preliminary phytochemical analysis of *M. linearis* using various solvents.

	Hexane	Ethyl acetate	Ethanol	Water
Alkaloids				
Dragendorff's test	-	-	-	-
Mayers test	-	-	-	-
Flavonoids				
Alkali test	-	++	+++	++
Glycosides				
Bornbager's test	+	+	+	+
Tannins				
Gelatin test	-	+	++	+
Saponins				
Frothing test	+	+	+	+
Terpenoids				
Nollers test	+	+	+	+
Anthraquinones	-	-	-	-
Benzene Ammonia Test	-	-	-	-
Phenols				
$FeCl_3$ test	-	++	+++	++

Therefore, this investigation reports on the antifungal activity of *Marchantia linearis* a bryophyte against selected pathogenic fungi.

Material and Methods

Plant material -Fresh thallus of *Marchantia linearis* was collected from Kallar river floor of Ponmudi hills, Kerala, India. Toxonomic identity was confirmed by comparing with authenticated herbarium specimen at Department of Botany Herbaria, Univesity of Calicut, Kerala. A voucher specimen of the plant is kept in the herbarium of the institute.

Test fungal samples -Fungi like *Candida albicans, Botrytis cinerea, Colletotrichum capsici, Fusarium oxysporum, Fusarium solani, Phytophthora capsici, Rhizoctonia solani* and *Sclerotinia sclerotiorum* were identified and procured from Institute of Microbial Technology (IMTECH-CSIR), Chandigarh, India.

Preparation of extracts-Fresh thallus (100 g) was chopped and successively extracted with 300 ml of hexane, ethyl acetate, methanol and water for 6 h by hot continuation extraction using soxhlet apparatus. The supernatants were concentrated using rotavapour at 50 °C. The yields of the extract were hexane (0.45 g), ethyl acetate (1.4 g), ethanol 8.5 g) and water 5.2 g), respectively. The ethanolic and water residues were lyophilized and stored at -20 °C.

Phytochemical screening : Various solvent extracts were subjected to various tests in order to detect the presence of different phytochemicals such as phenols, flavonoids, carbohydrates, glycoproteins, alkaloids, sterols and triterpenes outlined by Trease and Evans[5] and Harbone[6]. Further analyses were restricted to ethanol and water extracts.

Total phenols assay-Total phenols was determined using Folin Ciocalteu reagent at 765 nm and expressed in terms of gallic acid equivalent (mg/g of dry mass)[7].

Quantification of total flavonoids- Flavonoid was

Table 2. Growth inhibition of fungal pathogens by *M. linearis* ethanolic extract (EE); water extract (WE); fluconazole (FLU)(positive control).

Pathogens	% Growth inhibition	
	EE	WE
Candida albicans	96	65
Botrytis cinerea	82	62
Colletotrichum capsici	50	57
Fusarium oxysporum	97	64
Fusarium solani	48	60
Phytophthora capsici	90	62
Rhizoctonia solani	48	54
Sclerotinia sclerotiorum	47	60

quantified following the method of Chang *et al.*[8]. The calibration curve was prepared by quercetin at concentrations 12.5 to 100 µg/ml in methanol.

Antimicrobial assay

Determination of antifungal activity- The crude ethanol and water extracts were weighed and dissolved in a known volume of dimethyl sulphoxide (DMSO), to obtain a final concentration of 5 mg/ml. Antifungal actgivity was measured by a quantitative microspectrophotometric assay[9]. Growth inhibition was measured in 96-well microtiter plates at 595 nm. Routinely, tests were performed with 20 µl of the extract to be assayed, 10µl of a spore suspension and 70 µl of potato dextrose broth (PDB) (HiMedia). Microcultures containing 20µl of DMSO instead of test solution were used as a negative control. Fungicide fluconazole at 125 µg/ml to 1000 µg/ml was used as a positive control. The plates were lfet standing for 30 min at 30±2°C to allow the spores to sediment, after which absorbance was measured at 595 nm in a ELISA plate reader. After 48 h of incubation at 30±2 °C, growth was recorded by measuing absorbance. All assays for antifungal activity were carried out in triplicate. Growth inhibition[9] was determined based on the equation (ΔC-ΔT)/ ΔC] x 100, where ΔC is the corrected absorbance of the control microculture at 595nm and ΔT is the corrected absorbance of the test microculture. The corrected absorbance values equal the absorbance at 595 nm of the culture measured after 48 h minus the absorbance at 595 nm measured after 30 min.

Minimal inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) : The minimum inhibitory concentration (MIC) was determined by serial plate micro dilution method using serially diluted plant extracts according to the protocol of Eloff[10]. The ethanol and water extracts were diluted to get series of concentrations from 5 mg/ml to 100 mg/ml in sterile nutrient broth. The fungal suspension of 50 µl was added to the broth dilutions. These were incubated from 18 h at 37 °C. MIC of each extract was taken as the lowest concentration of the extract that inhibited any visible growth after 24 h of incubation. Fluconazole, tested at a concentation range of 125 µg/ml to 1000 µg/ml, were included as standards in each assay. Wells containing the ethanol, water, the solvent and DMSO employed in sample preparation were also included in order to monitor sample sterility and to determine any antifungal effects of the solvents. The microplates were incubated overnight at 30±2 °C for 48 h. As an indicator of fungal growth, 40 µl of *p*-iodonitrotetrazolium violet (INT), dissolved in sterile water, were added to the microplate wells and again incubated for at least 30 min to ensure adequate colour development. Since the colourless tetrazolium salt is

Table 3. Antifungal activity of various dilutions of different solvent extracts from *M. linearis.*

Pathogens	Ethanol extract							Water extract						
	1/2	1/5	1/10	1/15	1/20	1/40	1/100	1/2	1/5	1/10	1/15	1/20	1/40	1/100
Candida albicans	-	+	+	+	+	+	+	-	-	+	+	+	-	-
Botrytis cinerea	-	+	+	+	-	-	-	-	+	+	-	-	-	-
Colletotrichum capsici	-	+	-	-	-	-	-	-	+	-	-	-	-	-
Fusarium oxysporum	-	+	+	+	+	+	+	-	-	+	+	+	-	-
Fusarium solani	-	+	-	-	-	-	-	-	+	-	-	-	-	-
Phytophthora capsici	-	+	+	+	-	-	-	-	+	-	-	-	-	-
Rhizoctonia solani	-	+	-	-	-	-	-	-	+	-	-	-	-	-
Sclerotinia sclerotiorum	-	+	-	-	-	-	-	-	+	-	-	-	-	-

Ex : crude extract; + : Growth inhibition ≥ 85%; - Growth inhibition < 85%.

Table 4. Antifungal activity of ethanolic and water extracts of *M. linearis* against pathogenic fungi.

Pathogens	EE			WE			FLU	
	MIC	MFC	MFC/MIC	MIC	MFC	MFC/MIC	MIC	MFC
Candida albicans	250	1000	4	500	1000	2	250	500
Botrytis cinerea	125	500	4	500	1250	2.5	125	250
Colletotrichum capsici	1000	2500	2.5	1500	2000	1.3	500	1000
Fusarium oxysporum	125	500	4	500	1250	2.5	125	250
Fusarium solani	500	2000	4	1000	2000	2	250	500
Phytophthora capsici	250	1000	4	500	1000	2	125	250
Rhizoctonia solani	1000	2000	2	1500	2000	1.3	500	1000
Sclerotinia sclerotiorum	1500	2000	1.5	2000	2500	1.2	500	1000

Ethanolic extract (EE); water exract (WE); fluconazole (FLU) (positive control) Minimum inhibitory concentration (values in µg/ml); Minimum fungicidal concentration (values in µg/ml). Values are given as Mean ± SD (n=3) and considered to be significantly different at $P<0.05$.

reduced to a red coloured product by bilogically active organisms, the inhibition of growth could be visually assessed in the wells where the solution remained clear after incubation with INT. A clear solution or a definite decrease in colour reaction indicated inhibiton of growth. All data represent three replicate experiments per microorganism. The resultant MIC values were determined as the mean of these replicate experiments.

The *in vitro* fungicidal activity (MFC) was determined described by Espinel-Ingroff *et al.*[11]. After 72 h of incubation, 20 µl was subcultured from each well that showed no visible growth (growth inhibition of over 99.9%), from the last positive well (growth similar to that for the growth control well), and from the growth control (extract-free medium) onto PDA, plates. The plates were incubated at 27 °C until growth was seen in the growth control subculture. The minimum fungicidal concentration was regarded as the lowest extract concentration that did not yield any fungal growth on the solid medium used.

Results and Discussion

Phytochemical analysis- Preliminary phytochemical analysis showed the presence of carbohydrates, glycoproteins, flavonoids, alkaloids, sterols, phenols and triterpenes (Table 1). The ethanolic extract showed significant amount of total phenols (11.6 mg/g) and flavonoids 9.8 mg/g) compared to the water extract (total phenols-8.9 mg/g) and flavonoids (7.5 mg/g).

Antimicrobial activity- Antimicrobial assay exhibited different degree of growth inhibition against tested fungal species. Ethanolic and water extracts of *M. linearis* were screened *in vitro* for their antifungal activity against *Candida albicans, Botrytis cinerea, Colletotrichum capsici, Fusarium oxysporum, Fusarium solani, Phytophthora capsici, Rhizoctonia solani* and *Sclerotinia sclerotiorum.* The growth inhibtion for the crude extracts were measured (Table 2). Different resonse was shown by the extracts with the tested fungal species. Among the fungal pathogens evaluated, *Colletotrichum capsici, Fusarium solani, Rhizoctonia solani* and *Sclerotinia sclerotiorum* showed 47-50% inhibtion whereas, *Candida albicans, Botrytis cinerea, Fusarium oxysporum* and *Phytophthora capsici* showed above 82% inhibition values and are comparable with the commercial fungicide, fluconazole used as the positive control. *Candida albicans* and *Fusarium oxysporum* displayed an inhibition rates ranging from 96% to 97%. By contrast, *Sclerotinia sclerotiorum, Rhizoctonia solani* and *Fusarium solani* exhibited the lowest percentage of inhibitions. Interestingly, water extracts showed moderate inhibition with all fungi *i.e.,* 54-65%.

The effect of different dilutions of the plant extract against fungal species is summarized in Table 3. Ethanolic extract dilutions ranging from 1/2 to 1/15 inhibited growth of fungal sp. by more than 90%, while, the dilution factor for the water extract varied between 1/5 to 1/10.

M. linearis ethanolic extract expressed differential levels of fungistatic and fungicidal activites. The MIC and MFCs values ranged from 125 to 1500 µg/ml and from 500 to 2000 µg/ml respectively (Table 4). The lowest MIC (125 µg/ml) was shown by *Botrytis cinerea* and *Fusarium oxysporum* whiule highest MIC by *Sclerotinia sclerotiorum* (2000 µg/ml).

According to the results obtained, the ethanol seems to be more efficient in the exraction of bio molecules than water with antifungal activity. Meanwhile, water extract MIC and MFC values ranged from 500-2000 µg/ml and 1000-2500 µg/ml respectively. According to Berche *et al.*[12] the ratio MBC/MIC was used to evaluate antibacterial activity. If the ratio MFC/MIC=1 or 2, the effect was considered as fungicidal but if the ratio MFC/MIC= 4 or 16, the effect was defined as fungistatic. The data analysis indicates that the tested polyphenol extracts showed the significant results and are comparable with the fluconazole *i.e.* ethanolic extract showed fungistatic than fungicidal whereas water extract displayed fungicidal more prominently. The may be due to the fact that the bioactive constituents such as polyphenol compounds were responsible for the antimicrobial activity. In effect, some previous studies showed that polyphenolic compounds cause inhibition of a wide range of microorganisms. Phenol is well known as a chemical antiseptic[13]. In addition, phenolic and terpenic antimicrobial activities are well documented[13]. The antimicrobial activity of flavonoids is due to their ability to complex with extracellular and soluble protein and to complex with cell wall while that of tannins may be related to their ability to inactivate microbial adhesions, enzymes and cell envelop proteins[14].

Pinelo *et al.*[15], suggested that chemical characteristics of the solvent, the method used during the extraction process and diverse structural and compositional aspects of the natural products result in each material-solvent system showing distinct behaviour. Differences in polarity among various solvents have been reported to account for the differences in solubility of active plant active properties, hence variations in the degree of activity. The present sutdy is comparable with Dellavalle *et al.*[16] who reported 90% inhibition of *Alternaria solani* with *Rosmarinus officinalis* alcoholic

extract, whereas Itako *et al.*[17] showed 60% inhibition by *R. officinalis* with aqueous extract.

Comparision of the growth inhibition of the crude extracts and their respective dilutions shows a strong dependent effect on extract concentrations. In general, the fungicidal activity of extract dilutions is weaker compared to crude extracts. The present reslts revealed that antifungal activity of the crude extracts was enhanced by increasing the concentration of the extracts, in effect; the inhibition activity of the extracts was concentration dependent. The present finding is in agreement with the report of Banso *et al.*[18], who also observed that higher concentrations of antimicrobial substances showed more growth inhibition.

The antimicrobial activity of plant extracts might not be due to the action of a single active compound, but the synergistic effect of several compounds that are in minor proportion in the plant[19]. Crude plant extracts are generally a mixture of active and non-active compouds, and MICs of less than 500 µg/ml suggest good antimicrobial activity[4]. In this work, the MICs values showed variation, demonstrating significant to nomial antifungal activity against the fungal pathogens under study. Banso *et al.*[18] reported that the antifungal substances contained in the extracts were fungistatic at lower concentrations, while becoming fungicidal at higher concentrations of the extracts. The results of the present study indicate that the MFC of the extracts evaluated were obtained at similar or higher concentrations than in the MIC assays, but not at lower concentrations (Table 4). Plants do not contain an immune system and thus must rely on other mechanisms to defend themselves from pathogens or herbivores. In the case of fungal infection, these mechanisms include synthesis of bioactive organic compounds[20] or antifungal protein[21] or oxidative burst by reactive oxygen species. The quantity and quality of these active compounds depends on the plant species, plant tissue under study and environmental factors[4, 22].

In general, most of the ethanolic and aqueous extracts of the plants showed antifungal activities. This indicates the potential of *M. linearis* to be used as antimicrobial agents against fungal pathogens. However, further studies are warranted to screen the active principle compound in the extraction solvents and its toxicity to use them as sources and templates for the synthesis of drugs to control wound and other disease-causing fungi.

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