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ANTIFUNGAL ACTIVITY OF THE ETHANOLIC EXTRACTS OF *MARCHANTIA LINEARIS* LEHM AND LINDENB. AGAINST SOME PATHOGENIC FUNGI

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Abstract: Bryophytes belong to the group of the oldest known land plants. They are not damaged by fungi, bacteria and insect larvae because, biological compounds like phenylquinone, aromatic and phenolic substances, oligosaccharides, polysaccharides, sugar alcohols, amino acids, fatty acids, aliphatic compounds in bryophytes provide protection against these organisms, therefore, bryophytes have the potential for medical use. Although Bryophytes are very common, their medicinal importance is not exploited wholly. In this study, ethanolic extract of *Marchantia linearis* a bryophyte was evaluated *in vitro* for antifungal activities and their mode of action. Methodology includes hot continuous soxhlet extraction, RP-HPLC and HPLC-PAD, antifungal analysis by disc diffusion, microdilution assay, electron microscopy and isolation and assay of â-glucosidase, pectin lyase and protease. Screening of the phenols and flavonoids, revealed the presence of phenolic acids such as vanillate, gallate, coumarate, cinnamate, ferulate, para catechol, sinapate, hydroxybenzoate, chlorogenate and the flavonoids such as quercetin, luteolin and apigenin. Ethanolic extract exerted a potent fungicidal activity against *Botrytis cinerea and marginally in Rhizoctonia solani* i.e., showing varied levels of growth inhibition with different concentrations. Minimum inhibitory and minimum fungicidal potentialities showed varied response against the pathogen tested. Scanning and transmission electron microscopy examinations visualized changes like alteration in hyphal morphology and cell structure suggesting the fungistatic nature of the extract. Significant inhibition on the growth of the tested fungi and their hydrolytic enzymes, â-glucosidase, pectin lyase and protease suggest the multisite mechanism of action. The antifungal effects of the studied plant extract recommend them as potent candidate for the *in vivo* biological control.

Key words: Antifungal activity, Hydrolytic enzymes, *Marchantia linearis*, Phytochemical, Scanning electron microscopy, Transmission electron microscopy.

INTRODUCTION

World Health Organization stated that the infectious diseases remain the second leading causes of death worldwide. Plants harbour an inexhaustible source of active ingredients invaluable in the management of many intractable diseases from time immemorial (Menghani *et al*., 2011). India possesses a historical track record of significant global contribution by virtue of its traditional knowledge and genetic resources of medicinal plants (Bhagobaty *et al*., 2012). Several plants have been used in folklore medicine. The rational design of novel drugs from traditional medicine offers new prospects in modern healthcare. Pathogenic fungi are one of the main infectious agents and are indirectly responsible for allergic or toxic disorders among plants and animals (Dellavalle *et al*., 2011). Bryophytes have been used traditionally employed for treating various skin disorders and injuries. Recently from bryophytes, a pool of bioactive compounds is isolated and characterized with their pharmacological activity (Alam, 2012). *Marchantia* species had been used as herbal medicine in ancient China. Many bioactive components, particularly the conjugated polyphenols, have been isolated from many *Marchantia* species. The tribes of Wayanad *Kurichya, Kattunaikkans* use *Marchantia linearis* for curing skin borne allergies (Bhagobaty *et al*., 2012). Therefore, the present investigation was undertaken to evaluate the *in vitro* antifungal activities of ethanolic extract of *Marchantia linearis* Lehm & Lindenb. and their mode of action.

MATERIALS AND METHODS

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

Preparation of extracts: Fresh thallus (100 g) was chopped and successively extracted with 300 ml of hexane, ethyl acetate, ethanol 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 (0.451 g), ethyl acetate (1.4 g), ethanol (8.5 g) and water (5.2 g). The ethanolic and water residues were lyophilized and stored at -20°C.

Phytochemical screening: The different solvent extracts were initially subjected to qualitative analysis for secondary metabolites such as tannins, saponins, flavonoids, alkaloids, terpenoids and glycosides in accordance with Katasani (2011) and Chen et al. (2012) with little modification.

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) (Javed Kamal.2011).

Reverse Phase High Performance Liquid Chromatography (RP-HPLC) of phenols: Phenolic components of the extract were further fractionated following the method of Noumi et al. (2011) with appropriate standard phenolic acids. Phenolic acids in the sample were identified by comparing with the retention time of the standards.

Quantification of total flavonoids by Reverse Phase High Performance Liquid Chromatography (RP-HPLC) PAD: Flavonoids such as quercetin, (Q) luteolin (L), apigenin (A) were quantified by a PAD following RP-HPLC separation at 254.5 nm for Q, 345 nm for L and A. The chromatographic peaks of the analytes were confirmed by comparing their retention times and UV spectra with those of the reference standards. Quantification was carried out by the integration of the peak using external standard method.

Test fungal samples: Fungi like *Botrytis cinerea* and *Rhizoctonia solani* identified and procured from Institute of Microbial Technology (IMTECH-CSIR), Chandigarh, India.

Antimicrobial assay: Microdilution method was used to determine the minimum inhibitory concentration (MIC) and minimum killing concentration (MKC) of the crude ethanolic extract (Biswas et a., 2011). Fluconazole was used as positive controls.

Spore Germination and growth Kinetics Assay: The ethanolic extract (2 µL) was dissolved in 5% DMSO to obtain 31.25, 62.5, 125, 250, 500 and 1,000 µg/mLconcentrations of the extract, where the final concentration of DMSO was 0.5% (Bajpai and Kang, 2012). *Botrytis cinerea* appeared to be more sensitive fungi compared to *Rhizoctonia solani* to the extract in the spore germination assay.

Electron microscopy: Transmission (TEM) and scanning electron microscopy (SEM) was employed to analyze the mycelia treated with ethanolic extract and was compared with the untreated control.

Isolation and assay of b-glucosidase, pectin lyase and protease: b-glucosidase activity was assayed by using cellobiose as a substrate according to the method of Del Pozo et al.(2012). Pectin lyase activity was evaluated by the method of Al-Ajlani et al. (2012). Protease activity was determined according to Raut et al. (2012).

Statistical analysis: The data was statistically evaluated by one way ANOVA and t-test. The results are average of 6 replications and are represented as mean ± SD.

RESULTS

Phytochemical analysis: Phytochemicals screened from *M.linearis* thallus with various solvents showed the presence of alkaloids, flavonoids, phenols, glycosides and terpenoids (Table 1). Total phenol content (TPC) (11.6 mg/g) and flavonoids (9.8 mg/g) are significant in the ethanolic extract.

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	—	++	+++	++
Glycosies Bornbager's test	+	+	+	+
Tannins Gelatin test	—	+	++	+
Saponins Frothing test	+	+	+	+
Terpenoids Nollers test	+	+	+	+
Anthraquinones	—	—	—	—
Benzene Ammonia Test				
Phenols $FeCl_3$ test	—	++	+++	++

Fractionation of total phenols: Fractionation of phenols by RP-HPLC in *M. linearis* (Fig. 1) showed the presence of a pool of phenolic acids such as gallate (83.4 µg/g), vanilate (97.8 µg/g), chlorogenate (97 µg/g), cinnamate (98 µg/g), protocatechol (110.9 µg/g), coumarate (2001.3 µg/g), ferulate (23.7 µg/g), sinapic (1530.4 µg/g), caffeate (32.4 µg/g) and hydroxyl benzoate (0.9 µg/g). The retention time of standards and sample was listed in Table 2.

Quantification of flavonoids: Extraction of flavonoids with 80% ethanol under the frequency of 100 kHz, the tempera-ture of 25°C, the liquid-solid ratio of 10 mL/g and the time of 15 min, repeated thrice give the highest flavonoid yield (Fig. 2). Good results were obtained with respect to repeatability relative standard devia-tion (RSD) and recovery (97.27 - 99.68%). The contents of quercetin, luteolin and apigenin in *M.linearis* are 182.5, 464.5, and 297.5 µg/g respectively.

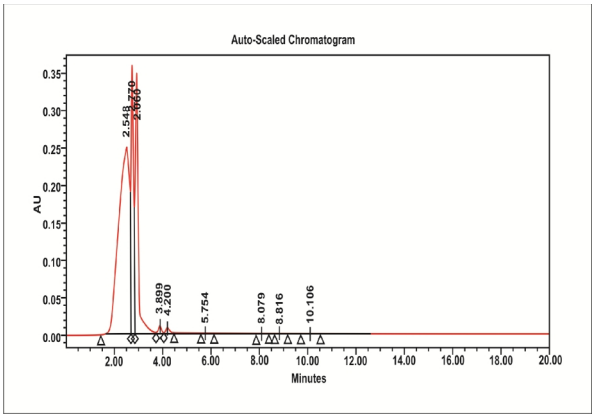

Fig. 1. Fractionation of phenols by RP-HPLC in *M. linearis*

Antimicrobial activity: Antifungal assay exhibited varied degree of growth inhibition against tested fungal species such as *Botrytis cinerea* and *Rhizoctonia solani* (Table 3). The microbicidal effect of the extract was further visualized as

Table 2. Comparison of retention time of standard phenolic acids with ethanolic extract of *M.linearis*

Phenolic acids	Retention time (Rt) in min.	
	standard	sample
Gallic acid	2.6	2.609
Vanillic acid	4.5	4.547
Caffeic acid	3.8	3.899
p-HBA	6.4	6.548
Ferulic acid	5.7	5.617
Sinapic acid	6.2	6.297
ProtoCatechol	3.9	3.899
Coumeric acid	7	7.072
Cinnamic acid	9.89	9.963
Chlorogenic acid	2.9	2.943

A gradient consisting of solvent A (2.5:97.5 v/v methanol-double distilled water at pH 3 with acetic acid) and solvent B (50:50 v/v methanol-double distilled water at pH 3 with acetic acid) was applied at a flow rate of 1mL/min. Injection volume of both the standards and the samples was 20 µ L.

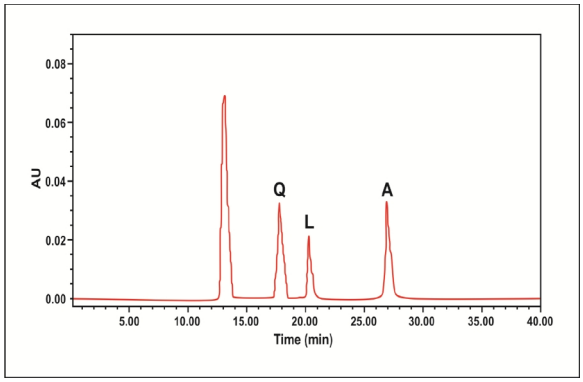

Fig.2. HPLC chromatograms using photodiode array detector (PAD) of flavonoids in the ethanolic extract of *M.linearis* (A): Q; (B): L; (C): A. Column: HIQ SIL C_{18}V. Mobile phase: methanol-acetonitrile-acetic acid-phosphoric acid-water (200:100:10:10:200, v/v/v). Flow-rate: 1 ml/min. Photodiode array detector at 254.5 nm for (a), 345 nm for (b)

inhibition zone by treating the pathogens with ethanolic extract and then spreading the cells on agar plates. Among the pathogens tested *Botrytis cinerea was* most sensitive *Rhizoctonia solani* the resistant species.

Ultra structural changes: Scanning electron microscopic (SEM) analysis showed remarkable morphological changes in treated *Botrytis cinerea*. The non treated hyphae displayed smooth surface with uniform width and straightly elongated branches. Meanwhile, ethanolic extract treated hyphal filaments were characterized by irregular branching, swollen tips, distortion, collapse and/or shrinkage with wrinkled rough surfaces (Fig. 3 a & b). Control *B. cinerea* cells possess many mitochondria with normal size and shape, were scattered throughout the cytoplasm (Fig. 4a) but, the cytoplasm of the treated cells showed abnormal vacuolization and degenerated mitochondria (ruptured outer membranes, degeneration of mitochondrial cristae, matrix which showed variation in electron density) in the TEM analysis. Similarly, alteration in the endomembrane system, including release and breaking up of the plasmalemma from the cell wall and partial dissolution of the nuclear envelope and the rough endoplasmic reticulum (Fig. 4b).

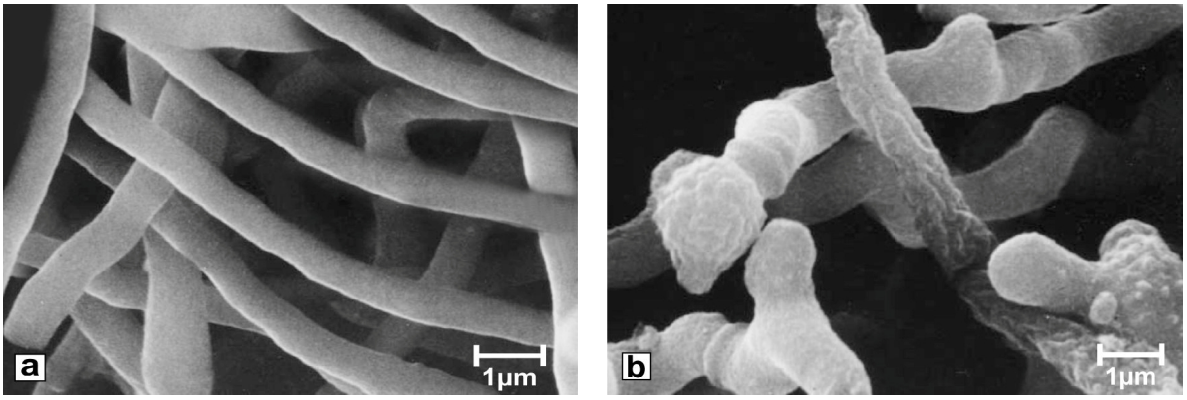

Fig. 3a & b. (a) Hyphae of *Botrytis cinerea* showing normal filaments with smooth surfaces (control) (b) condensed hyphal branches with a rough, blebbed and wrinkled surface

Table 3. 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
Botrytis cinerea	125	500	4	500	1250	2.5	125	250
Rhizoctonia solani	1000	2000	2	1500	2000	1.3	500	1000

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

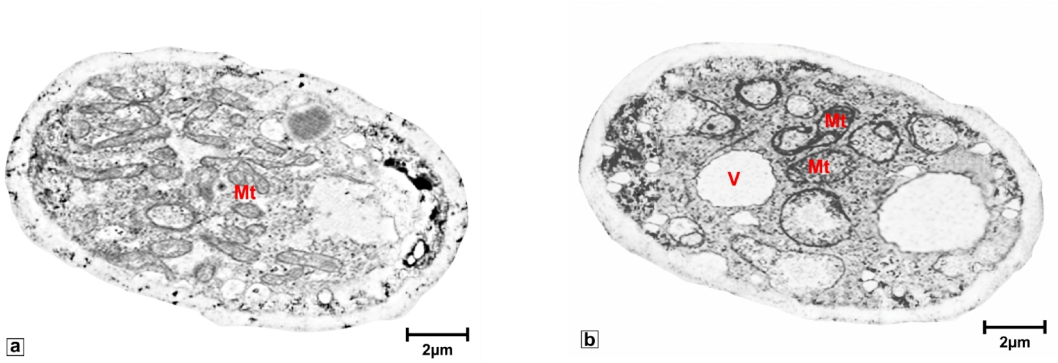

Fig. 4a & b. (a) TEM micrograph of *B. cinerea* control cells showing many normal mitochondria scattered throughout the cytoplasm (b) ethanolic extract treated - large anomalous vacuole in the cytoplasm, degenerated mitochondria and disrupted cell wall

Spore germination and growth kinetics: DMSO (0.5%, v/v) as a negative control did not inhibit the spore germination of any of the fungal pathogens tested. 80% inhibition of fungal spore germination was observed for *B. cinerea* at 250-500 µg/mL concentrations of extract. Similarly, ethanolic extract also exhibited a moderate inhibitory effect on the spore germination of *R. solani* in the range of 20 to 60% at concentrations ranging from 500 to 1,000 µg/mL.

The antifungal kinetics of the ethanolic extract against sensitive fungal species spores to different concentrations of the extract for a period of 0 to 150 min caused varying degrees of inhibition of spore germination. A positive correlation was observed between concentration and exposure time with growth kinetics among the fungal spores tested. An increase in fungicidal activity was observed with increase in exposure time and concentration. The extract at 31.25 µg/mL showed antifungal activity but not rapid killing at the exposure time of 120 min. However, there was a remarkable increase in the killing rate at 250 and 500 µg/mL after 30 min of exposure, and 60% to 90% inhibitions of spore germination were observed at 150 min exposure, respectively.

Profile of hydrolytic enzymes: The effect of the ethanolic extract on the production of major hydrolytic enzymes such as â-gulcosidase, pectin lyase and protease from *the fungi* were analyzed. â-gulcosidase were significantly inhibited by the ethanolic extract of *M.linearis* in *Botrytis cinerea* (1.7 - 0.2 U/ mg protein) at all the tested concentrations (250-1000 µg/mL) compared with control. However, the corresponding activity in *Rhizoctonia solani* was ranging from 2.6 - 0.28 U/ mg protein i.e., a moderate inhibitory effect (Table 4). Similarly, pectin lyase from *Botrytis cinerea* was remarkably inhibited at all tested concentrations and moderately inhibited in *Rhizoctonia*. Protease also showed a similar pattern in *Botrytis cinerea* followed by *Rhizoctonia solani* at all the tested concentrations.

Table 4. The effect of ethanolic extract from *M.linearis* with 3 different concentrations (250, 500, 1000 ì g/mL) on the production of â-glucosidase (BG), pecin lyase (PL) and Protease (PR) (U/ mg protein) from the selected pathogenic fungi

	250			500			1000		
	BG	PL	PR	BG	PL	PR	BG	PL	PR
B. cinerea	1.7±0.002	0.6 ±0.03	0.4±0.01	0.9±0.01	0.3±0.02	0.4 ±0.04	0.4±0.001	0.28 ±0.02	0.2±0.03
R. solani	2.6±0.04	0.7 ±0.03	0.5±0.04	1.4±0.02	0.5±0.03	0.4±0.02	0.8±0.05	0.3 ±0.02	0.28±0.09

Values are given as Mean ± SD (n= 3) and considered to be significantly different at $P < 0.05$.

DISCUSSION

In this study, we have demonstrated morphological and ultra structural changes of selected pathogenic fungi affected by ethanolic extract of *M.linearis* under varying treatment conditions. The results obtained in the present study revealed that the level of the phenolic compounds in the ethanolic extract of the thallus in *M. linearis* were significant ($P<0.05$). Values of phenolic acids retention time were in agreement with those published by Noumi *et al.* (2011). A positive correlation was observed between the phenolic acids and total phenols in the bryophyte suggesting their role as precursor of many of the phytochemicals. Cinnamate, coumarate, gallate, ferulate and hydroxy benzoate has established antioxidant potentiality, which inturn supports the antioxidant significance of the plant. Chlorogenic acid can regenerate oxidized vitamin E via caffeate and it also acts as a pro oxidant in the propagation phase of LDL oxidation. Coumarate is a precursor of flavonoids and also binds with nitric acid and its derivatives before they combine with protein amines to form nitrosamine radical. Similarly cinnamate has antibacterial, antifungal and antiparasitic properties and gallate and its derivatives exhibit higher free radical scavenging properties (Rajbir Kaur *et al.*, 2011).

Protective effect of the *M. linearis* extract was comparable to the methanolic extract of the liverwort *Plagiochila beddomei* (Manoj *et al.*, 2011). Antimicrobial activity of polyphenolic compounds is likely exerted primarily by its ability to act as a nonionic surface-active agent therefore disrupting the lipid-protein interface or by the denaturation of proteins and inactivation of enzymes in the pathogens. Secondly, phenols alter the permeability of the membrane that could result in the uncoupling of oxidative phosphorylation, inhibition of active transport and loss of metabolites due to membrane damage. Gallic acid has proven anti-fungal and anti-viral properties (Rajbir Kaur *et al.*, 2011).

Treatment with ethanolic extract caused irregular branching in the apical region, loss of linearity, with the appearance of barrel-like structures followed by extrusion of amorphous fibrillar substance with vesicular apical surface. Growth of fungi takes place at the apex of terminal hyphae (Vijayarathna, 2012), so the abnormalities observed at this region after treatment suggests the inhibition of fungal growth. The changes in the wall surface indicate an alteration in the normal assembly of the wall components, leading to their incorrect arrangement at the apical dome and to the anomalous lateral exocytosis from the basal hyphae. Anomalous blebbing on the apex, extrusion of material suggests an alteration in the normal control of apical exocytosis of vesicles carrying material for cell wall construction compounds, such as chitin, glucans, and glycoproteins in the apical region of fungi (Latha *et al.*, 2011).This leads to incorrect arrangement of the different parietal wall compounds.

The results suggest that the cell membrane is the target of the polyphenols that could interfere with the phospholipid bilayers of membranes, as previously observed by Belay *et al.* (2011) on isolated bacterial cytoplasmic membrane and also in fungal cells by Latha *et al.* (2011). However, the interaction of the lipophilic compounds with the cytoplasmic membrane also depends on the presence of water-soluble compounds like terpenoids (Afifi, 2012). Thus, phenolic acids or flavonoids induce its fungicidal activity by targeting cell membranes. Since the regulation of the main metabolic systems depends on the integrity of all the cell organelle membranes, it is clear that the presence of compounds which affect cell membranes may interfere with many biological processes; in particular, any alteration of the mitochondrial membrane system can reduce energy turn over inside the cell and can result in premature senescence. Afifi (2012) reported *Anethum graveolens*, *Cymbopogon citrates* and *Juniperus oxycedrus* extracts against *Fusarium oxysporum*, *Aspergillus niger*, *Alternaria alternate*. So the present results strongly suggest that the hyphal degeneration observed in *B. cinerea* may be due to the phytochemicals in the ethanolic extract of *M.linearis*. Morphological and ultrastructural changes of growing hyphae as well as of constituting hyphal cells affected by *M.linearis* extract under varying treatment conditions suggest that the polyphenols inhibit the cell wall components. Therefore, it is the rational speculation that inhibition by the extract of fungal cell wall can induce abortive cell-wall formation in susceptible fungi. Actually, the primary morphological changes produced by growth-inhibitory concentrations are those

associated with the inhibition of normal cell wall formation. One of the most prominent changes was the development of numerous short branches on the lateral walls of hyphae. Although the exact reason remains to be answered, this abnormal hyphal growth might be a fungal response that compensates the inhibited apical growth of hyphae. Similar morphological changes were reported previously by Afifi (2012) on selected fungal pathogens.

Similarly, antifungal kinetics results using *Cestrum nocturnum* plant extract were also in strong agreement with the findings of Al-Reza *et al*. (2010) on *in vitro* and *in vivo* plant pathogens. The present data on the differential rate of inhibition on β-gulcosidase, pectin lyase and protease suggests that the pathogenic fungi possess a specific infection pattern in order to enter the host plant. This is usually performed by the synthesis of a characteristic set of polymer-degrading enzymes such as cellulases and pectin-degrading enzymes to enable success-ful establishment of the host-pathogen relationship. Most of the compatible pathogens possess multiple forms of these cell wall degrading enzymes (Arzoo *et al.*, 2012). The results of the present study related with the inhibi-tory effect from natural plant extracts against the hydrolytic enzymes was supported by Sofia *et al*. (2012) on *Botrytis cineria* using methanolic and ethanolic fraction of three native Chilean plants (*Ephedrabreana*, *Fabiana imbricata,* and *Nolana sedifolia*). Similarly, Jaroszuk-Sciseand Kurek (2012) isolated water-soluble substances from the leaves of rye were shown to inhibit the fungal pectinase and cellulase. Also, Akhgari *et al.* (2012) reported that the bean polygala-cturonase-inhibiting protein expressed in transgenic *Brassica napus* inhibits polygalacturonase from its fungal pathogen *Rhizoctonia solani*. Similarly, Pedro *et al.* (2011) reported that the *Carica papaya* leaf extract possess different inhibitory effects against *Phomopsis vexans, Fusarium sp, Rhizoctina solani, Sclerotiurn rolfsii, Rhizopus stolonifer, Colletotrichum gloeosporioides* and *Phytophthora capsici* hydrolytic enzymes.

CONCLUSIONS

In conclusion, the results showed that fungicidal effects of biologically active compounds from *M.linearis* thallus ethanolic extract can be used as good candidates for the *in vivo* biological control of pathogenic fungi, limiting the abuse of chemical fungicides. The morpho-functional disintegrity of fungal cell components and reduction in the activities of hydrolytic enzymes may be the probable mechanism of action leads to non-viability and poor spore germination capacity. More studies are warranted in the future to test the antifungal activities of the studied plant extracts on other fungi.

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