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INVESTIGATIONS ON ANTIFUNGAL ACTIVITY OF *ATRICHUM UNDULATUM* (HEDW.) P. BEAUV AGAINST *ALTERNARIA ALTERNATA* AND *FUSARIUM OXYSPORUM*

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ABSTRACT

The present study evaluated the activity of various solvent extracts of *A. undulatum* against *Alternaria alternata* and *Fusarium oxysporum*. Both the phytopathogenic fungi showed good sensitivity against all the extracts of plant. As methanol extract showed maximum antifungal activity and maximum total phenolic content as well as total flavonoid content, therefore, it was selected in further investigations. FTIR analysis of methanol extract showed presence of various functional groups like phenols, flavonoids, ketones, terpenoids and alcohols responsible for antifungal potential. GC-MS also revealed the presence of eleven biologically active compounds, including Stigmasterol and 5,8,11,14- Eicosatetraenoic acid, methyl ester with antifungal activity. FE-SEM analysis demonstrated the damaging effect of methanol extract on the cell wall of fungal hyphae. Hence, *A. undulatum* depicts potential as an eco-friendly substitute for the harmful synthetic fungicides.

Key words : Methyl ester, Methanol extract, Flavonoid content, *Atrichum undulatum*, *Alternaria alternata*.

Introduction

Phytopathogenic fungi have significantly impacted the agriculture sector, by seriously impairing the crops during their growing and harvesting seasons, resulting in low-quality grains, fruits and vegetables as well as upto 30% reduction in worldwide yields. Fungal pathogens selected for present study *Alternaria alternata* and *Fusarium oxysporum* are more prevalent among the hundreds of soil borne microorganisms that cause diseases in a wide range of plant species. Many *Fusarium* spp. induce wilting in a variety of crops, including tomatoes, watermelon, lentils, and bananas (Bertoldo *et al*., 2015). Likewise, *Alternaria alternata* has been reported to cause leaf spot and other serious damage in about 400 different plant species (Meena *et al.*, 2017). It affects a wide range of hosts, causing blights, rots, and leaf spots.

Nowadays, chemical fungicides are primarily used to control fungal infections (Zhu, 2020). The extensive use of chemical fungicides, however, has resulted in numerous issues, including ecological imbalances, environmental pollution and serious impact on human health. With their more frequent use the pathogens develop resistance also to chemical fungicides and lose susceptibility to them (Zhang, 2019). It has prompted to explore an alternative approach for reducing dependency on synthetic fungicides.

Plant extracts possess potent antimicrobial properties and could be a feasible alternative to commercial fungicides (Javaid and Iqbal, 2014; Javaid and Rauf, 2015). Their non-toxic nature and biodegradability have opened up a new vista of sustainability. Many secondary metabolites produced by plants including flavonoids, phenols and phenolic glycosides, unsaturated lactones, sulfur compounds, saponins, cyanogenic glycosides, glucosinolates and tannins have already proved to display

antifungal potential (Dissanayake and Jayasinghe, 2013; Vinale *et al*., 2014).

Bryophytes are the world's oldest known terrestrial plants. They are the second most diverse group of green plants after angiosperms, contributing significantly to overall biodiversity. They lack protective shields as bark but have a variety of bioactive compounds that protect them from hostile environments. Almost all the bryophyte species are not damaged by fungi, bacteria, or insect larvae because biological compounds in bryophytes such as phenylquinone, aromatic and phenolic substances, oligosaccharides, polysaccharides, sugar alcohols, amino acids, fatty acids and aliphatic compounds provide them protection against these organisms (Asakawa, 1982; Askawa, 1988; Asakawa, 1994; Asakawa, 1999; Asakawa, 2007 and Asakawa, 2017).

Several bryophytes, namely, *Asterella angusta, Atrichum undulatum, Bryum argenteum, Dumortiera hirsuta, Fontinalis antipyretica, Marchantia polymorpha, Pallavicinia lyellii, Physcomitrella patens, Plagiochasma intermedium, Plagiochila fasciculata, Scapania terrucosa* and *Tortella tortuosa* are reported with antifungal activities against different fungal pathogens (Dey and De, 2011). Among them, the moss *Atrichum undulatum* was reported with various biological activites like antimicrobial, anti-inflammatory and antifungal activity. Glime (2007) reported the use of *Atrichum* in the Chinese medicines in antibacterial and anti-inflammatory formulations. Saxena and Yadav (2018) investigated the antifungal potential of *Atrichum undulatum* against the fungi *Fusarium oxysporum* and *Aspergillus fumigatus* and antibacterial potency against the five bacteria *Escherichia coli, Bacillus mycoides, Proteus mirabilis, Staphylococcus aureus* and *Salmonella typhi*. All the microbes showed good sensitivity except *A. fumigates* and *P. mirabilis.*

The phytochemicals extracted from plants would be an excellent alternative to fungicides being both safe and biodegradable. Present study was undertaken to investigate the effect of the moss *A. undulatum* extracted in different solvents against two phytopathogenic fungi *Alternaria alternata* and *Fusarium oxysporum* for the first time. The active phytochemicals present in the methanolic extract responsible for antifungal properties were also investigated.

Materials and Methods

Plant material

Atrichum undulatum (Hedw.) P. Beauv. was collected from around the Wildflower Hall, An Oberoi Resort of Shimla district of Himachal Pradesh. The plant material was identified by morphological characters using keys by Kashyap (1932a, 1932b) and Singh and Singh (2009) and deposited in Department of Botany, Panjab University, Chandigarh under PAN No. 6427.

Test pathogens

Alternaria alternata (MTCC- 6572) and *Fusarium oxysporum* (MTCC- 284) were procured from Microbial Type Culture Collection and Gene Bank of Institute of Microbial Technology (IMTECH), Chandigarh, India and maintained on potato dextrose agar (PDA) medium.

Preparation of Plant Extract

Fresh thalli were washed thoroughly with tap water followed by distilled water to remove all the contaminants, then shade dried at room temperature and finally homogenized to a fine powder. 5gm of powdered plant material was then subjected to extraction in 150ml of each solvent by using Soxhlet apparatus. The solvents used for extraction were 99.65% Acetone, 99.5% Methanol, 95% Hexane and 99% Di-ethyl ether. The solvents were heated to their boiling temperatures i.e., acetone (56°C), methanol (64.7°C), hexane (68.7°C) and di-ethyl ether (34.6°C) and the extraction continued for 6–8 h at 2–4 cycles h^{-1}. The extracts were then dried and stored at 4ºC until use. Five mg of each extract dissolved in 1 ml of 10% DMSO was used for antifungal assay.

Antifungal assay

Antifungal activity of the different extracts of *M. polymorpha* was determined by Poisoned Food Technique (Shivapratap *et al.*, 1996) against the selected fungal strains. Poisoning was done by putting five-day old fungal culture discs on the agar plate prepared by impregnating with 1 ml of plant extract with concentration of 5mg/ml. The plates were then incubated at 28 ± 2 °C for five days. Percent inhibition (PI) of mycelial growth was evaluated by comparing the colony diameter of the poisoned plate (with plant extract) and non-poisoned plate (control with 10% DMSO) and calculated using the formula given below:

$$\% \text{ Inhibition} = \frac{[\text{Mycelial growth (control)} - \text{Mycelial growth (poisoned)}]}{\text{Mycelial growth (control)}} \times 100$$

Phytochemical analysis

Total phenolic content : Total Phenolic Content (TPC) was quantified in each extract, following the protocol of Bray and Thorpe (1954). 0.5 ml of each extract was added to 0.5 ml of Folin- Ciocalteu reagent and 1.0 ml of distilled water. After 2-5 minutes, 0.5 ml of 10% Na_2CO_3 was added to the tube. After 1 hr incubation

at room temperature, the absorbance was measured on a spectrophotometer UV-VIS spectrophotometer at 760 nm, using distilled water as a blank. Gallic acid was used as the standard and total phenol content was expressed as mg Gallic Acid Equivalents per gram (GAE g^{-1}) of the sample in dry weight (mg g^{-1}).

Total flavonoid content : The Total Flavonoid Content in different extracts of *A. undulatum* was determined by using aluminium chloride method (Sembiring *et al*., 2018). 50 µl of the each extract (5mg/ml) was added to 10 µl of 10% aluminium chloride solution and followed by 150 µl of 95% ethanol. 10 µl of 1M sodium acetate was added to the contents. 80% ethanol was used as reagent blank. All reagents were mixed and incubated for 40min at room temperature and protected from light. The absorbance was measured at 415nm. Quercetin was used as the standard and total flavonoid content was expressed as mg Quercetin Equivalents per gram (QE g^{-1}) of sample in dry weight (mg g^{-1}).

FTIR analysis : Fourier transform infrared (FTIR) spectroscopic technique was used to identify the presence of functional groups in the most potential methanolic *extract*. FTIR spectrum of was recorded on Bruker Tensor 27 FTIR instrument using KBr pellet Attenuated Total Reflectance (ATR) or Diffuse Reflectance (DR) in the wavelength range of 600–4000/ cm^{-1}.

GC-MS: For the phytochemical analysis of selected methanol extract, Nucon 5765 equipped with a standard capillary column was used. Helium was used as a carrier gas with a flow rate of 1 ml min^{-1}. Column oven temperature was set at 50°C for 2 min, and then increased to 300°C for 10 min at 5°C min^{-1}. The volume injected was 2 µl, keeping split ratio 10:1, solvent cut time 4.50 min, ion source temperature 200°C and interface temperature 260°C. The compounds were identified by comparing Retention Time (RT) and mass spectra with NIST20M1 mass spectral library.

Effect on the cell membrane of fungal hyphae

Field–Emission Scanning Electron microscopy : For FE-SEM observations, the method described by Wu *et al*. (2023) was adopted with minor modifications. Mycelial blocks (4.0 mm × 4.0 mm) were cut from each fungus plate, which served as control and from the plates poisoned with the plant extract at the concentration of 5mg/ml. Each fungal sample was treated with 4% glutaraldehyde for 4 h. After treatment, the sample was washed three times by keeping in 0.01 M PBS for 15 min each. Then the fungal sample was dehydrated in different concentration of ethanol *i.e*. 20%, 50%, 80% and 100 % ethanol for 15 min each. After dehydration, it was freeze-dried in vacuum freeze-dryer for 2 h and gold was sprayed by ion sputtering apparatus. Finally, the fungal sample was observed under Field- Emission Scanning Electron Microscope (JSM-6100).

Statistical analysis

All the above experiments were performed in triplicates. Data were expressed as Mean ± SEM; n=3 and evaluated by One-way analysis of variance (ANOVA), followed by Turkey's multiple comparisons test using Graph Pad Prism (ver.8.0.1).

Results

Antifungal activity : The plant extracts of *A. undulatum* showed good antifungal activities against the presently selected phytopathogens *Alternaria alternata* (with percent inhibition of 53.33±0.68, 52.16±0.68, 43.92±1.36 and 38.43±0.68 in methanol, acetone, di-ethyl ether and hexane extracts, respectively) and *Fusarium*

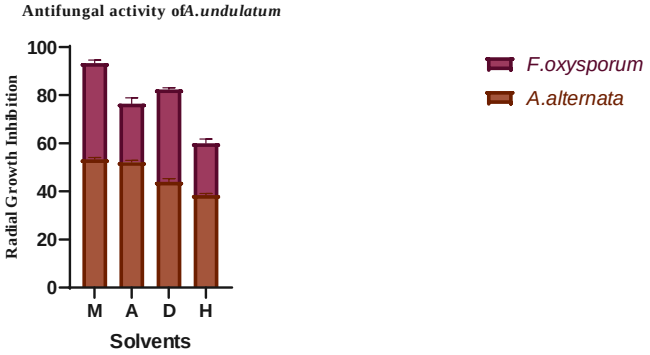


Fig. 1 : Histogram showing antifungal activity of Acetone(A), Methanol(M), Hexane(H) and Di-ethyl ether(D) extracts of *A. undulatum* against two pathogenic fungi. were expressed as Mean ± SEM; n=3 and evaluated by One-way analysis of variance (ANOVA), followed by Turkey's multiple comparisons test.

Table 1 : Total Phenolic Content and Total Flavonoid Content of *A. undulatum*. (One-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test showed * p < 0.0001 compared to Total Phenolic Content of Acetone, Di-ethyl ether and Hexane plant extracts. # p <0.0001 compared to Total Flavonoid Content of Acetone, Di-ethyl ether and Hexane plant extracts. GAE: Gallic Acid Equivalent; QE: Quercetin Equivalent).

Plant extracts	Total Phenolic Content (in mg GAE g^{-1})	Total Flavonoid Content (in mg QE g^{-1})
Methanol	97.33±1.27*	91.17±1.99#
Acetone	72.41±2.16	59.77±0.25
Hexane	43.9±0.65	20.37±0.81
Di-ethyl ether	89.56±0.71	54.50±1.14

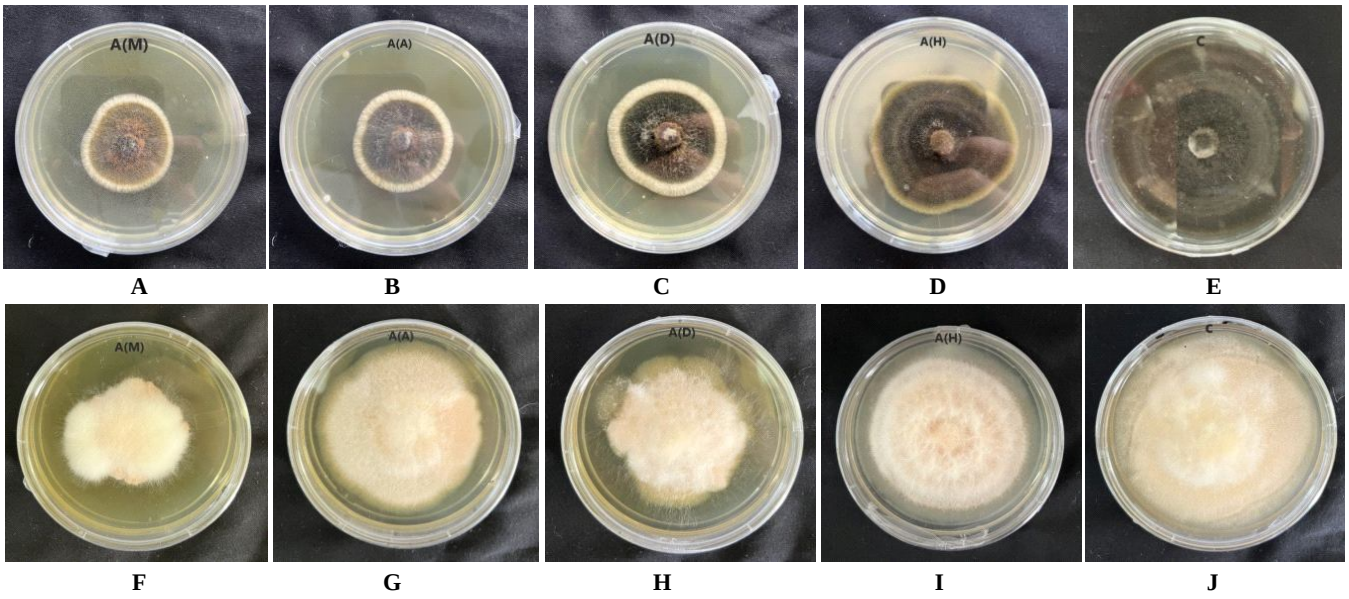


Fig. 2 : Plates showing antifungal activity of *A.undulatum* extracted in four different solvents: Methanol(M), Acetone(A), Di-ethyl ether(D) and Hexane (H) with their respective controls (C) against *Alternaria alternata* (A-E), against *Fusarium oxysporum* (F-J).

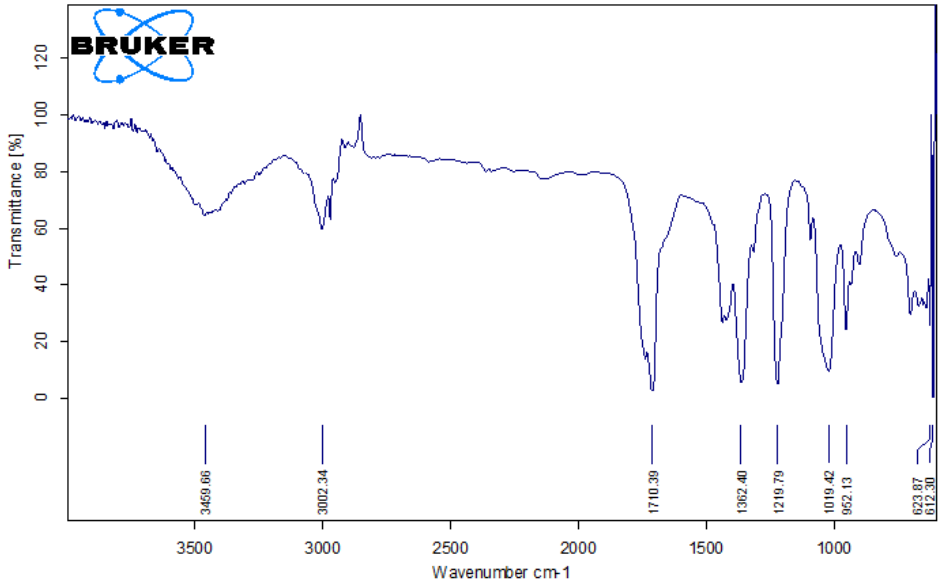


Fig. 3 : FTIR spectral analysis of methanol extract of *A. undulatum.*

oxysporum (with percent inhibition of 40.00±1.18, 23.53±0.68, 38.43±0.68 and 21.18±00 in methanol, acetone di-ethyl ether and hexane extracts respectively) as shown in Figs. 1, 2. Since, the maximum antifungal activity of methanol extract was observed, the methanol extract was further used to study the presence of bioactive compounds responsible for antifungal properties.

Phytochemical analysis

Total Phenolic Content (TPC) and Total Flavonoid Content (TFC) : Total Phenolic Content was found maximum in plant's methanol extract (97.33±1.27 mg GAE g^{-1}) followed by in its di-ethyl ether extract (89.56±0.71mg GAE g^{-1}) and in acetone extract (72.41±2.16mg GAE g^{-1}), while it was found minimum in hexane extract (43.9±0.65mg GAE g^{-1}). Similarly, Total Flavonoid Content was also found to be highest in methanol extract (91.17±1.99mg QE g^{-1}), followed by in acetone extract (59.77±0.25 mg QE g^{-1}) and in di-ethyl ether extracts (54.50±1.14mg QE g^{-1}) , and lowest in hexane extract (20.37±0.81mg QE g^{-1}).

FTIR analysis

The FT-IR spectra of the methanol extract of *Atrichum undulatum* is shown in Fig. 3. It revealed peaks in 952.13 cm^{-1} to 3459.66 cm^{-1} range, corresponding to several functional groups. The absorption peaks at 3459.66 cm^{-1} and 3002.34 cm^{-1} are due to an O-H functional group of the hydroxyl group, which is related with alcohols and phenols. The peak at 1710.39 cm^{-1} attributed to C=O stretching suggested the existence of simple carbonyl compounds such ketones, aldehydes, esters and carboxyl. The next peak, at 1362.40 cm^{-1}, represents O-H stretching, which indicates the presence of phenols or tertiary alcohols. Furthermore, the absorption band at 1219.79 cm^{-1} was ascribed to the C = O stretching that corresponds to vinyl ether, whereas the peak at 1019.42 cm^{-1} was assigned to the C-O stretching that indicates the presence of primary alcohol. Thus, FTIR

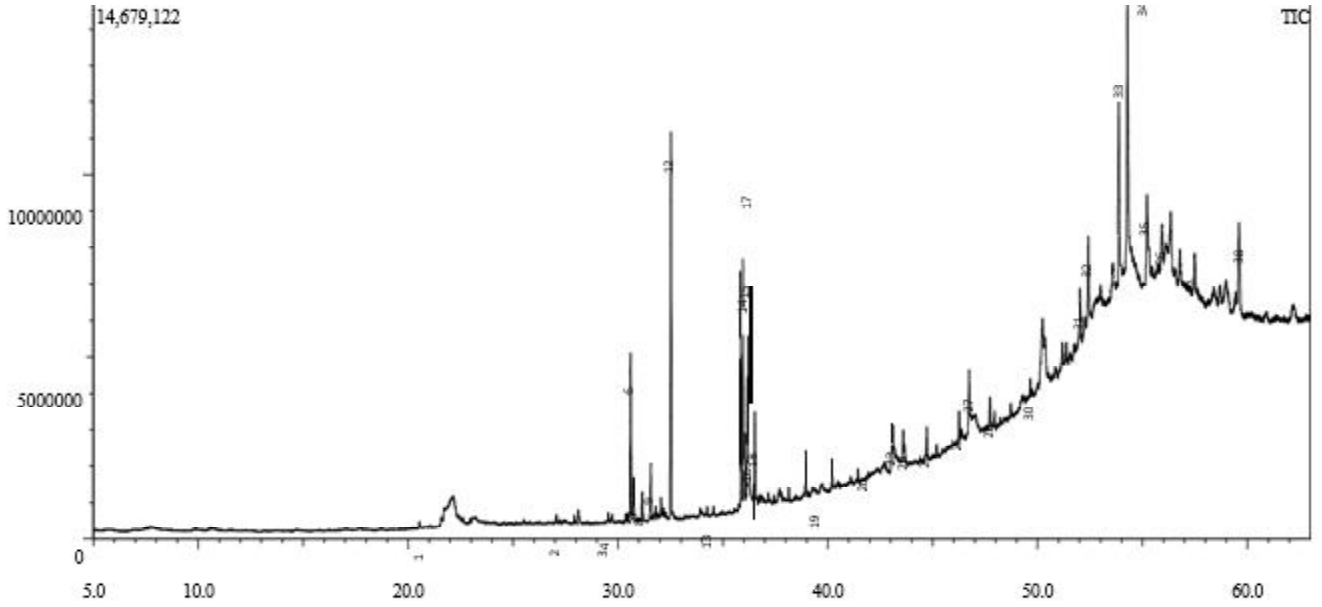
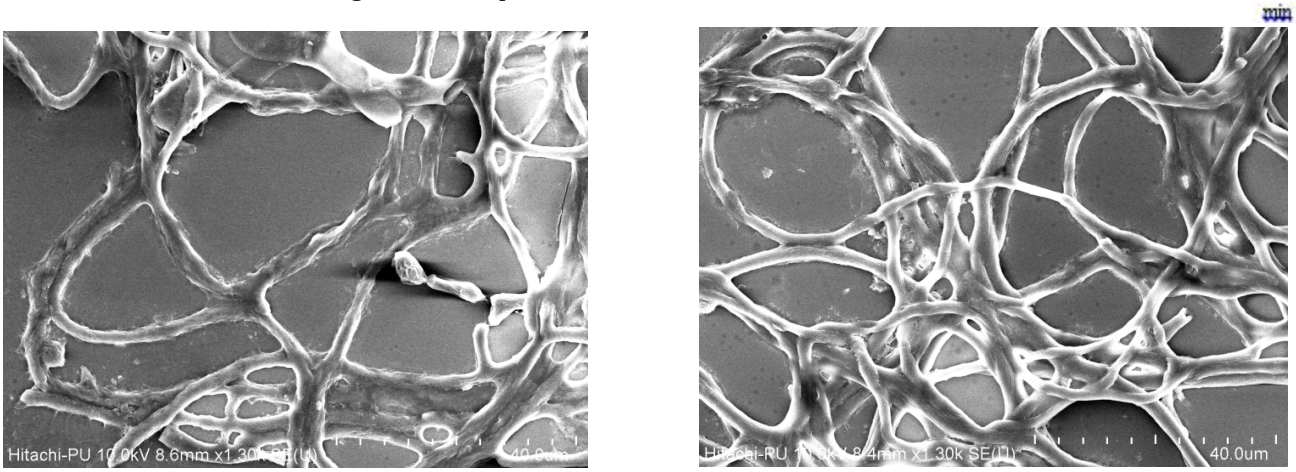
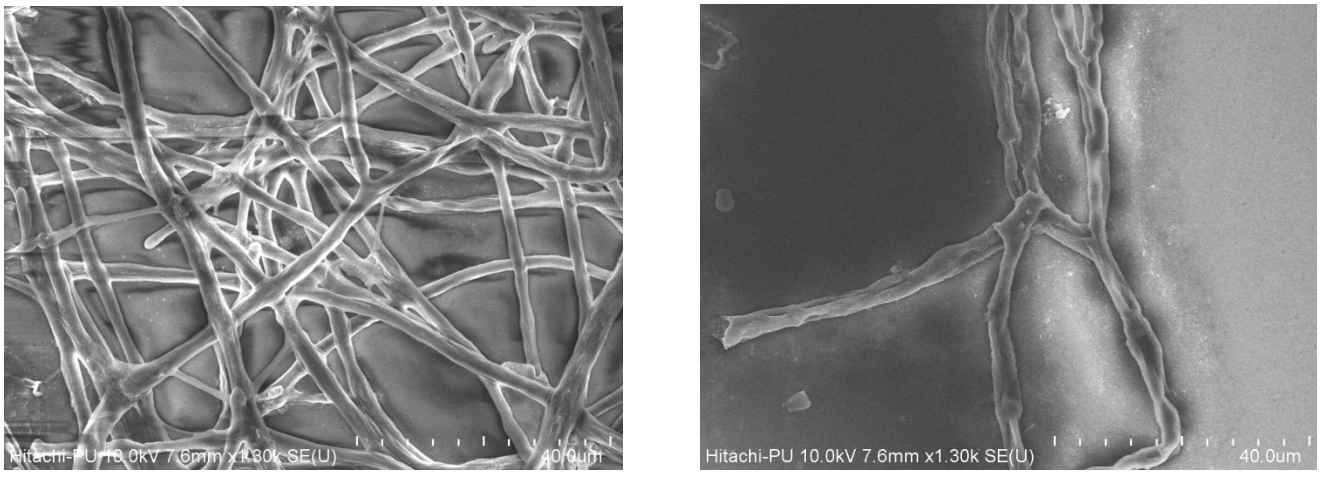


Fig. 4 : GC-MS profile of methanol extract of *A. undulatum.*



A) **B)**

Fig. 5 : FE-SEM images of fungal hyphae of *A. alternata* A) Control B) After treatment with methanol extract of *Atrichum undulatum.*



A) **B)**

Fig. 6 : FE-SEM images of fungal hyphae of *F. oxysporum* A) Control B) After treatment with methanol extract of *Atrichum undulatum.*

Table 2 : GC-MS Tentative identification of compounds present in methanol extract of *A. undulatum* with their biological activities.

S. no.	Proposed Compound	Retention Time	Peak Area %	Molecular Formula	Molecular Weight	CAS No.	Biological Activity
1	Phytol	36.239	11.45	$C_{20}H_{40}O$	296	150-86-7	Cytotoxic, Anti- inflammatory, Anti-microbial and Anti-oxidant actitvity (Islam *et al*., 2018)
2	Stigmasterol	54.286	10.59	$C_{29}H_4O$	412	83-48-7	Anticancer, anti-inflammatory, anti-diabetic, antifungal, antibacterial, antioxidant, and neuro protective properties (Bakrim *et al*., 2022)
3	Campesterol	53.914	8.87	$C_{28}H_{48}O$	400	474-62-4	Anti inflammatory, antidiabetic and anticancer activities (Miras-Moreno *et al*., 2016)
4	Quercetin	59.604	4.46	$C_{30}H_{50}O_7Si_5$	662	4067-66-7	Antioxidant, anti-inflammatory, antibacterial, antiviral, radical-scavenging, gastroprotective and immune-modulatory activities (Kim and Park, 2018)
5	Gamma-Sitosterol	55.220	3.09	$C_{29}H_{50}O$	414	83-47-6	Anti inflammatory, antidiabetic and anticancer activities (Miras-Moreno *et al*., 2016)
6	dl-.alpha.-Tocopherol	52.420	2.94	$C_{29}H_{50}O_2$	430	10191-41-0	Antidiabetic, Antiarthritic, Anti-cancerous, Anti-infertility, Antioxidant (Duke, 2017)
7	Methyl stearate	36.529	2.57	$C_{19}H_{38}O_2$	298	112-61-8	Antioxidant activity (Mazumder *et al*., 2020)
8	Methyl lignocerate	46.736	1.70	$C_{25}H_{50}O_2$	382	2442-49-1	Anti- diabetic property (Shilpa, 2009)
9	4-Campestene -3-one	55.934	1.69	$C_{28}H_{46}O$	398	51014-22-3	Not found
10	beta.-Sitosterol acetate	52.021	1.62	$C_{31}H_{52}O_2$	456	915-05-9	Anti inflammatory, antidiabetic and anticancer activities (Miras-Moreno *et al*., 2016)
11	5,8,11,14-Eicosatetraenoic acid, methyl ester, (all-Z)	38.942	1.37	$C_{21}H_{34}O_2$	318	256689-4	Antifungal, antibacterial, antitumor cytotoxic effects (Agoramoorthy *et al*., 2007)

analysis confirmed the presence of various functional groups.

GC-MS analysis

The bioactive compounds present in the methanolic extract of *Atrichum undulatum* were tentatively identified by using GC-MS. The active principles with their retention time (RT), molecular formula, molecular weight (MW), concentration (peak area %) are presented in Table 2, which shows the presence of 11 bioactive phytochemical compounds in the methanolic extract of *Atrichum undulatum*. The mass spectra of identified compounds from *Atrichum undulatum* were presented in Fig. 4. Among the identified phytocompounds, the major compounds with reported antimicrobial activity are Phytol, Stigmasterol, Quercetin and 5,8,11,14-Eicosatetraenoic acid, methyl ester.

FE-SEM

As discussed above, the methanol extract of *A. undulatum* strongly inhibited the growth of *A. alternata* and *F. oxysporum*, FE-SEM confirmed the inhibitory

effect of methanol extract on both these fungi. The mycelia of *A. alternata* after treatment with methanol extract showed irregular shapes and loss of symmetry with distorted apices as compared with the control sample which showed homogeneous cell structure and normal mycelia with smooth apices. Some swellings were also observed on the treated mycelia and their apices (Fig. 5). Similar distortions were observed in the mycelium of *F. oxysporum* when treated with methanol extract. The untreated mycelium was regular, plump and smooth, and contained full cytoplasm. After treatment the mycelium became severely exfoliated and shriveled, with a bursting and wrinkled surface (Fig. 6).

Discussion

Antifungal activity of the moss *Atrichum undulatum* in four different solvents (Acetone, Methanol, Di-ethyl ether and Hexane) against two phytopathogenic fungi *A. alternata* and *F. oxysporum* was studied for first time by Poison Food Technique. *A. alternata* and *F. oxysporum* were found highly sensitive to all the extract of *A. undulatum.* Previously, Sabovljevic *et al*. (2011) investigated the antifungal activity of two mosses (*Atrichum undulatum* and *Physcomitrella patens*) and a liverwort (*Marchantia polymorpha*), grown naturally and in laboratory conditions (*in vitro*) against other five fungal species (*Aspergillus versicolor, A. fumigatus, Penicillium funiculosum, P. ochrochloron* and *Trichoderma viride*). In comparison of naturally grown bryophytes, the extracts made from axenically farmed bryophytes were found more active. All the fungi were found sensitive against the di-ethyl ether extract of selected bryophytes. Antifungal activity of *A. undulatum* against six other fungal strains *Candida albicans, C. glabrata, C. tropicalis, C. parapsilosis, Issatchenkia orientalis* and *Kluyveromyces marxianus* was studied by Kaur *et al*. (2023). The butanol extract showed maximum antifungal activity against *K. marxianus, I. orientalis, C. tropicalis* and *C. parapsilosis* and methanol extract against *Candida albicans* and *C. glabrata*. In the present study, the methanol extract showed maximum antifungal activity. The phytochemical analysis also showed highest TPC (97.33±1.27 mg GAE g^{-1}) and TFC (91.17±1.99 mg QE g^{-1}) in the methanolic extract accounting for its good antifungal activity as compared to other extracts of *A. undulatum.*

FE-SEM studies were used to reveal the damaging effects of potent plant extract on fungal mycelial cells. This study is the first attempt to observe morphological changes in cell wall of *A. alternata* and *F. oxysporum* by methanol extract of *A. undulatum*. The methanol extract of *A. undulatum* caused deformations and distortions in fungal mycelium resulting in cell wall damage. Earlier, Otibi and Rizwana (2019) reported bioactive compounds in the leaf extracts of an angiosperm *Artemisia sieberi* which showed degenerating effects on the cell wall morphology of *Alternaria alternata, Fusarium moniliforme* and *F. oxysporum*. Various bioactive phytocomponents like phenols and flavonoids, which are lipophilic and quickly diffuse across the cell membrane suppressing the fungal growth effectively. They also interfere with the formation of important cell membrane constituents like glucans, ergosterol and mannans. This interference alters the cell structure by disrupting the membrane system, targeting its stability, causing disintegration, eventually leading to cell death (Yusoff, 2020).

The FTIR analysis of methanol extract presently revealed the presence of various functional groups like O-H, C=O, C-O confirming the presence of phenolic compounds, flavonoids, alcohols and terpenoids. The GC-MS analysis also revealed the presence of various bioactive compounds with reported biological activity. The major compounds identified in the present investigations are Phytol, Stigmasterol. Campestrol Quercetin, gamma-Sitosterol, Tocopherol, 5,8,11,14- Eicosatetraenoic acid, methyl ester etc. Of these 5,8,11,14- Eicosatetraenoic acid, methyl ester (Agoramoorthy *et al.*, 2007) and Stigmasterol (Bakrim *et al.*, 2022) are reported to have antifungal activity. Based on all these investigations, the moss *A. undulatum* comes out to have a great potential as an ideal ecofriendly alternative for controlling phytopathogenic fungi effectively.

Conclusion

Use of plant extracts against plant pathogenic fungi is an important field of study and would be a best alternative to existing chemical fungicides. The present study clearly indicates the efficacy of *A. undulatum* in controlling or preventing the growth of fungal phytopathogens, due to the presence of various bioactive phytocompounds responsible for the damage of the fungal cell structure by disrupting the cell membrane, which eventually leads to the cell death. Therefore, *A. undulatum* can serve as an alternative to chemical fungicide in the agriculture sector.

Statements and Declarations

No potential conflict of interest was reported by the author(s).

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