Research Paper

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ANTIMICROBIAL ACTIVITIES OF ORGANIC SOLVENT EXTRACTS OF EQUISETUM HYEMALE (L.)

BOTANY

KEYWORDS : *Equisetum hyemale*, organic solvent extracts, antimicrobial activities, MIC, MBC.

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ABSTRACT

Three organic solvent (petroleum ether, chloroform and methanol) extracts of Equisetum hyemale (L) have been tested against ten fungal species and four bacterial species for their antimicrobial activities. Of the three extracts, petroleum ether extract showed antifungal activities against four fungal species (Aspergillus niger, Curvularia lunata, Penicillium expansum and Trichoderma viride) and methanol extracts showed antifungal activities against Drechslera oryzae only. However, all the three extracts showed significant antibacterial activities against the tested bacterial species (Staphylococcus aureus, Enterococcus faecalis, Escherichia coli and Pseudomonas aeruginosa).

INTRODUCTION

Equisetum hyemale (L.) belonging to the family Equisetaceae is a pteridophytic perennial plant with branching rootstocks rooted at the internodes and growing upto 1 m in height. The aerial stems are cylindrical, hollow, simple and covered with silica. The cones are terminal. They prefer shady moist *soils. Equisetum* is the only surviving genus of Sphenopsida (Gierlinger *et al*., 2008). The genus has a history stretching back to the Crustaceous and possibly as far back as the Triassic and perhaps be the oldest living genus of vascular plants (Hauke, 1978). The *genus consists* of species which are often used in traditional medicine systems. *Equisetum arvense is* reported to possess antinociceptive, anti-inflammatory, antidiabetic, antioxidant or free radical scavenging, antimicrobial, anti-haemorrhagic, astringent, anticonvulsive, cytotoxic, diuretic, hepato-protective, vasorelaxant, wound healing and platelet anti-aggregant properties. The stems of *E. hyemale* have been used as diuretic, diaphoretic, astringent homeostasis and for treatment of eye diseases (Park and Tomohiko, 2011). They are also reported to exhibit antioxidant activity (de Queiroz *et al*., 2015a). Further, they are also highly efficient in treating urinary tract infection, cardiovascular diseases, respiratory tract infection and medical skin conditions (Sinha, 1996). Phytochemistry of the plant also shows the presence of minerals like silicic acid and silicates, potassium, calcium, aluminium, sulphur, magnesium and manganese, phenolic acids, phenolic pterosins, phenolic glycosides, triterpenoids, alkaloids, saponins, phytosterols, branched and long chain dicarboxylic acids and other constituents like true proteins and enzymes (mainly thiaminase) (Sandhu *et al*., 2010). Phytochemical study using high resolution Raman imaging reveals the presence of silica, pectin, hemicelluloses (glucomannan) and cellulose from the shoots of *E. hyemale* (Gierlinger *et al*., 2008). Six compounds from the stem of *E. hyemale* were also isolated and based on the spectral evidence, their structures were elucidated as trans-feruloyl-4-β-glucoside, cis-feruloyl-4-β-glucoside, transcaffeoyl-3-β-glucoside, kaempferol-3-sophoroside, kaempferol-3-sophoroside-7-β-glucoside, and herbacetin-3-sophoroside-8-β-glucoside (Park and Tomohiko, 2011). Presence of such secondary metabolites is a clear indication of the *E. hyemale* to possess antimicrobial property. But studies regarding antimicrobial activities of *E. hyemale* are lacking so far. The present paper reports the antifungal and antibacterial activities of three organic solvent (petroleum ether, chloroform and methanol) extracts of the shoots of *E. hyemale* against 10 fungal species and 4 bacterial species.

MATERIALS AND METHODS

Plant Material

Plants of the *E. hyemale* were collected from Manipur University campus. The plant was identified and voucher specimen of it was deposited to the Herbarium, Department of Life sciences, Manipur University. The accession number allotted to the plant is 001290. Healthy and matured parts of the test plant were collected, separately washed with tap water and cut into small pieces, dried in the shade and powdered with the help of a blender. The powdered plant material was filled in the thimble and extracted successively with three organic solvents viz., petroleum ether (PE), chloroform (CH) and methanol (ME) using a Soxhlet extractor at 40-60^{0}C. All the extracts were concentrated using rotary flash evaporator, collected separately and preserved at 4^{0}C in airtight brown bottles until further use. These extracts were used for assaying *in-vitro* antimicrobial activities against the test microorganisms.

Test fungal species

Nine plant pathogenic fungal species and one biocontrol agent were selected as the test fungi for the present study. The phytopathogenic fungal species were *Alternaria alternata* (Fr.) Keissler (leaf spot of beans), *Alternaria solani* Sorauer. (early blight of potato and tomato), *Aspergillus flavus* Link. Syn. *A. humus* Abbot. (aflatoxin contamination of food and grains), *Aspergillus niger* Van Tieghem.(black mold disease of fruits and vegetables), *Curvularia lunata* (Wakker) Boedijn. (grain discoloration of rice), *Drechslera oryzae* Breda de Haan (brown spot disease of rice.), *Fusarium oxysporum*(Schl.) emend. Snyder & Hansen. (wilt of tomato), *Penicillium expansum* Link ex. Fries (soft rot disease of fruits), *Penicillium italicum* Wehmer (blue mould rot of citrus fruits) and the biocontrol fungal agent was *Trichoderma viride* These fungal species were isolated from the respective diseased plant materials using standard mycological techniques. *Trichoderma viride* was isolated from field soil. The isolate showed antagonistic activity against *Fusarium oxysporum*. Stock cultures of these fungal species were maintained on Czapeck Dox Agar (CDA) medium.

The identities of some of the fungal cultures were confirmed at ITCC, IARI, New Delhi and NFCCI, Pune. They were *Alternaria alternata* ITCC Id no. 8246.11, *Aspergillus flavus* NFCCI 2791, *Aspergillus niger* ITCC Id no. 8241.11, *Drechslera oryzae* ITCC Id no. 8240.11, *Fusarium oxysporum* NFCCI 2790 and *Trichoderma viride* ITCC Id no. 8229.11.

Test bacterial species

The bacterial strains used for experiments include two Gram-positive bacteria *Staphylococcus aureus* (ATCC 25923) and *Enterococcus faecalis* (ATCC 29212) and two Gram-negative bacteria *Escherichia coli* (ATCC 25922) and *Pseudomonas aeruginosa* (ATCC 27853). The cultures of the bacterial strains were obtained from the Department of Microbiology, Regional Institute of Medical Sciences (RIMS), Imphal, Manipur, India. The bacterial cultures were maintained on nutrient agar medium (NA) at

37^{0}C and sub-cultured periodically. Stock cultures of the bacteria were also maintained on semi-solid agar at 4^{0}C for further use.

Screening for antifungal activity

The *in-vitro* antifungal activity was carried out by following the poisoned food technique as described by Nene and Thapliyal (1979). In the preliminary screening each extract was first dissolved in dimethyl sulphoxide (DMSO) and amended to CDA medium to get three final concentrations of 0.1, 0.5 and 1.0mg/ml of the culture medium. In each sterile Petri plate (9 cm dia) 25ml of CDA medium amended with a plant extract having a particular concentration was poured. After solidification of the medium, an agar culture disc (5mm dia), aseptically removed from 2-3 days old culture of a test fungus, was placed at the center of each plate. Three replicated plates were maintained for each treatment. The inoculated plates were incubated at 25^{0}C for 72 hours. The radial growths of the test fungi were measured and the percent growth inhibition was calculated using the following formula (Vincent, 1947):

$$I = \frac{(C - T)100}{C}$$

Where, I= percent growth inhibition

C= Colony diameter in control medium – culture disc diameter (mm)

T = Colony diameter in treated medium– culture disc diameter (mm)

After the preliminary screening concentration of 0.5mg/ml was found to be suitable and further assessments of antifungal activity were performed using this concentration.

Screening for antibacterial activity

Antibacterial activities of the plant extracts were determined using disc diffusion method. The bacterial culture grown in NA medium was suspended in fresh normal saline. The turbidity of the resulting suspension was adjusted to 0.5 McFarland turbidity standard. Sterile Mueller Hinton Agar (MHA) plates were swabbed with the culture of the respective bacterial species using sterile cotton swabs and kept for 15 min in laminar chamber for absorption to take place. The sterile filter paper (Whatmann No.1) discs (6mm dia) were impregnated with 20µl of a plant extract solution to achieve desired concentration of 1mg plant extract/disc and placed on the inoculated agar plates. Standard antibiotic streptomycin (10µg/disc) was used for comparison with the plant extract treatment. Five replicates were maintained for each treatment. The antibacterial assay plates were incubated at 37^{0}C for 24h and the mean diameters (mm) of the inhibition zone were recorded.

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

Determination of MIC was carried out using the broth dilution method (Oyeleke *et al*., 2008). One ml of reconstituted plant extract solution having a concentration of 100 mg/ml was added to another test tube containing 1 ml of sterile broth so as to obtain a concentration of 50 mg/ml. One ml of this dilution was transferred to another test tube in the same manner till the 8th test tube was reached. The 9th test tube did not contain any extract, but a solution of pure solvent served as negative control. Then 1 ml of an 18h old culture of each of the bacteria earlier adjusted at 10^{8}cfu/1ml was inoculated into each tube and thoroughly mixed on a vortex mixer. The tubes were incubated at 37^{0}C for 24h and observed for bacterial growth in form of turbidity. The test tube with the lowest dilution with no detectable growth by visual inspection was considered the MIC.

The MBC values were determined by removing 0.10 ml of bacterial suspension from the MIC tubes that did not show any growth and sub-cultured in MHA plates and incubated at 37^{0}C for 24 hr. The concentration at which no visible growth was observed was recorded as the MBC.

RESULTS AND DISCUSSIONS

The antifungal activity of the organic solvent extracts of *E. hyemale* is shown in Table 1. In the present investigation it was found that all the three extracts were either ineffective or showed negligible antifungal activity against the test fungal strains. Out of the three organic solvent extracts, only the PE extract and the ME extract showed antifungal activities of which PE extract was found to be more effective. The PE extract of *E. hyemale* was effective against four fungal species. The four species with their respective percentage inhibition were *C. lunata* (15.93%), T. Viride (4.31%), *P. expansum* (2.50%) and *A. niger* (1.45%).The ME extract of *E. hyemale* was found to be effective against only *D. oryzae* showing inhibition percent of 4.60%. The CH extract could not show growth inhibition against any of the tested ten fungal species. Rather, growth stimulation was observed in those fungal species where the extracts were ineffective. Similar observations were also reported by Palanichany and Nagarajan (1990) and Bajwa *et al.* (2004) where fungal growth was stimulated by plant extracts.

Table 1: Effect of organic solvent extracts of *Equisetum hyemale* (at 0.5mg/ml conc.) on radial growth of different fungal species.

Sl. No.	Fungal species	Radial growth (mm)			
		Petroleum ether	Chloroform	Methanol	Control
1	Alternaria alternata	28.33(-)	36.00(-)	36.00(-)	23.33
2	Alternaria solani	30.67(-)	45.67(-)	44.33(-)	27.67
3	Aspergillus flavus	32.33(-)	37.33(-)	42.33(-)	28.67
4	Aspergillus niger	27.67(1.45)	37.00(-)	52.67(-)	28.00
5	Curvularia lunata	36.67(15.93)	48.00(-)	48.67(-)	42.67
6	Fusarium oxysporium	36.00(-)	39.00(-)	37.33(-)	35.67
7	Drechslera oryzae	35.33(-)	34.33(-)	32.67(4.60)	34.00
8	Penicillium expansum	18.00(2.50)	22.33(-)	23.33(-)	18.33
9	Penicillium italicum	13.67(-)	15.67(-)	15.33(-)	12.33
10	Trichoderma viride	86.33(4.31)	90.00(-)	90.00(-)	90.00

*Values in parenthesis indicate percent inhibition over control.

(-) indicates no growth inhibition.

Table 2: Antibacterial activity of different solvent extracts of *Equisetum hyemale* (1mg/disc).

Organism (bacteria)	Zone of inhibition(mm)			
	Petroleum ether	Chloroform	Methanol	Streptomycin (10µg/disc)
E. coli	7.2 ± 0.45	7.0 ± 0.71	7.2 ± 0.45	14.8 ± 0.84
E. faecalis	13.2 ± 2.35	13.2 ± 1.48	12.4 ± 2.41	12.2 ± 2.95
P. aeruginosa	9.2 ± 0.84	8.8 ± 0.45	8.8 ± 0.45	10.2 ± 0.45
S. aureus	10.2 ± 0.45	7.6 ± 0.55	6.8 ± 0.84	16.4 ± 0.55

	Organism (bacteria)	Solvent extracts	Organism x Solvent extracts
	0.40	0.40	0.80
CD (0.05)	0.80	0.80	1.59

Values are means of 5 replicates ±SD

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Table 3: MIC and MBC values (mg/ml) of effective plant extracts of *Equisetum hyemale* against susceptible bacterial species.

Bacterial species	PE		CH		ME	
	MIC	MBC	MIC	MBC	MIC	MBC
E. coli	1.56	1.56	3.13	3.13	1.56	3.13
E. faecalis	1.56	1.56	3.13	6.25	3.13	6.25
P. aeruginosa	3.13	3.13	6.25	6.25	6.25	6.25
S. aureus	0.39	0.78	1.56	3.13	3.13	6.25

The antibacterial activities of the three organic solvent extracts of *E. hyemale* against the four bacterial species are shown in Table 2. All the three solvent extracts were found to inhibit the growth of all the four bacterial species. The zones of inhibition induced by the extracts against the test organisms ranged from 6.8mm to 13.2mm. PE extract of *E. hyemale* was found to be most effective in inhibiting the growth of all the tested bacterial species. The inhibition zone against *E. faecalis* (13.2mm) was the widest followed by *S. aureus* (10.2mm). For CH extract the inhibition zones ranged from 7.0mm to 13.2 mm.The inhibition zones induced by ME extract ranged from 6.8mm to 12.4mm. The zone of inhibitions observed in *E. faecalis* for the three extracts were even greater than that formed against the standard antibiotic streptomycin.The MIC value of *E. hyemale* extracts ranged from 0.39mg/ml to 6.25mg/ml. The MBC values were in concentration range of 0.78mg/ml and 6.25mg/ml.The present study showed that the three extracts of *E. hyemale* possessed potent antibacterial activity. Ferrazzano *et al.* (2013) also showed strong antibacterial activity of *E. hyemale*. Uzun *et al.* (2004) examined petroleum ether and ethanol extracts of *E. telmateia* against different microorganisms by disc-diffusion method and reported that petroleum ether extracts showed certain activity on *S. aureus, S. epidermidis* and *Candida albicans*. Milovanović and coworkers (2007) also tested hydro-alcoholic extract of *E. telmateia* against certain fungi and bacteria and reported that the extracts showed good antimicrobial activity against Gram-negative bacteria. Further, they reported that the extracts had little influence on fungi. Our present finding suggests the extracts of *E. hyemale* to be effective against both Gram-positive and Gram-negative bacteria with highest inhibition against *E. faecalis*. Radulović *et al.* (2006) reported that 1:10 dilution of the essential oil of *E. arvense* possess a broad spectrum antimicrobial activity against all tested bacterial species (*S. aureus, E. coli, Klebsiella pneumoniae, P. aeruginosa* and *Salmonella enteritidis*). In another finding, Radojevic *et al.* (2012) showed that the extracts of *E. telmateia* possess significant antibacterial activity against Gram-positive bacteria and weak to moderate activity against 7 fungal species. de Queroz *et al.* (2015b) evaluated the antimicrobial activity against oral microorganisms and the *in vitro* and *in vivo* toxicity of 70% ethanol and methanol extracts of *E. hyemale* and reported that both extracts presented low cytotoxicity even in high concentrations and the 70% ethanol extract of *E. hyemale* did not present toxicity inducing significant alterations and/or death in mice. It is evident from the present study that the organic solvent extracts of *E. hyemale* have antibacterial and antifungal activities. This finding along with the findings of previous workers suggests *E. hyemale* extracts may have beneficial therapeutic applications in bacterial infectious diseases.

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