Study of antimicrobial activity and root symbionts of *Hemionitis arifolia*

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Published online: 11 May 2011

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Abstract Antibacterial and antifungal activity of crude extract, alcoholic extract and extracted phenol from various parts of tropical pteridophyta, *Hemionitis arifolia* were tested by agar diffusion and tube dilution assay. Both the crude and alcoholic extracts of vegetative and reproductive leaves of *H. arifolia* showed considerable antibacterial activity against Gram negative test strain of *Escherichia coli* (MTCC-739). Extract from reproductive leaves also showed moderate antibacterial activity against *Bacillus subtilis* (MTCC-441) (Gram positive test strain) but didn't show any antifungal activity against *Candida albicans* (MTCC-7353). Mycorrhizal and other symbiotic association with the root system of *H. arifolia* was studied and it is revealed that a number of mycorrhizal strains were present in both vegetative and reproductive form. Presence of Dark Septate Endophytic Fungi (DSF) was also detected.

Keywords Antimicrobial activity · Dark Septate Endophytic Fungi (DSF) · Extract · *Hemionitis arifolia* · Mycorrhiza

Introduction

Human being is exploiting pteridophyta as a source of medicinal plants since time immemorial. *Hemionitis arifolia* (*H. arifolia*), an herbaceous member of Family – Hemi-onitidaceae occurs very rarely in South India (Moore 1859), Manipur, Nagaland, Mizoram, Assam, and Tripura (Ghosh et al. 2004). For the first time we have collected this plant from some pocket of Hooghly District (Barakpoor), West Bengal.

It grows on damp, shady, warm (20 °C–40 °C) place; mainly found in close vicinity of bamboo bushes. It has anti diabetic effect, studied in mice system (Nair et al. 2006) but no antimicrobial activity of *H. arifolia* is reported yet. The juice of its frond is also applied to burns (Ghosh et al. 2004). Our aim of the present work is to study antimicrobial activities of *H. arifolia* and its associated root symbionts.

Materials and methods

For the study of antimicrobial activity of *H. arifolia* crude extracts from different plant parts (vegetative leaf, reproductive leaf, vegetative stem, reproductive stem and rhizome) were prepared by crushing 1 g each of fresh sample with mortar and pestle and extracted in 80 % boiled ethanol. This ethanolic mixture was then centrifuged at 4,000 rpm for 10 min. Then the supernatant were taken and the total volume of each sample was made to 5 ml with 80 % boiled ethanol. Then 4 ml distilled water was added to each of these alcoholic extracts and were kept on hot plate at 40 °C to evaporate the alcohol. Thus the crude extract comes in water solution (Obongoya et al. 2010). For each extraction of plant part, six replicates were made.

In the second set of experiment alcoholic extract from different plant parts (vegetative leaf, reproductive leaf, vegetative stem, reproductive stem and rhizome) of *H. arifolia* were prepared by crushing 1 g each of fresh samples with mortar and pestle in 5 ml 80 % ethanol. These ethanolic extracts were then distilled by distillation apparatus and collected in separate vials (Singh et al. 2008). For each extraction of plant part, six replicates were made.

Total phenol content of various parts (vegetative leaf, reproductive leaf, vegetative stem, reproductive stem and rhizome) of *H. arifolia* was estimated by Folin Ciocalteu's method (Bray and Thorpe 1954). 50 mg of each sample was

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Table 1 Agar cup assay of crude extracts against *E. coli*

Samples	Diameter of zone of Inhibition (mm)
Vegetative Leaf	10
Reproductive leaf	12
Vegetative Stem	0
Reproductive Stem	0
Rhizome	0

Table 2 Agar cup assay of alcoholic extracts against *E. coli*

Samples	Diameter of zone of Inhibition (mm)
Vegetative Leaf	14
Reproductive leaf	15
Vegetative Stem	0
Reproductive Stem	0
Rhizome	0

homogenized in 10 ml of 80 % ethanol and agitated for 15 min at 70 °C; filtered through muslin cloth and again through Whatman No.1 filter paper and the volume of the filtrate was adjusted to 5 ml with 80 % ethanol. In a test tube 1 ml of ethanol extract, 1 ml of Folin Ciocalteu reagent and 2 ml of 20 % sodium carbonate solution were added and the mixture was heated in a boiling water bath for a minute. Then the tube was cooled under running tap water. After cooling, the volume was made up to 25 ml with distilled water. A reagent blank was maintained with 1 ml of distilled water instead of plant extract. The intensity of colour was read at 650 nm in a colorimeter. The amounts of total phenols present in samples were calculated from a standard curve prepared by using different concentrations of phenol. For this analysis six replicates were made.

Antimicrobial activities of various types of extracts were measured using two different methods viz Agar cup assay (Vesterdal 1946) and Tube dilution technique (Murray et al. 2003). In agar cup assay two bacterial strains viz *Bacillus subtilis* (MTCC-441), *Escherichia coli* (MTCC-739) and one fungal strain, *Candida albicans* (MTCC-441) were used. Cups of 6 mm diameter were made on the solidified, inoculated agar plates in a systematic manner with cork borer. 0.1 ml of different extracts (crude, alcoholic and phenolic) from various plant parts (vegetative leaf, reproductive leaf, vegetative stem, reproductive stem and rhizome) of *H. arifolia* were applied in separate cups with controls (water, 80 % ethanol and phenol). Plates were

Fig. 1 Tube dilution graphs against *E. coli* MTCC-739 (a & b), *B. subtilis* MTCC-441 (c & d)

incubated at 37 °C for 24 h. After 24 h diameter of hallow zones formed due to cell lysis surrounding each cup were measured. In each of these experiments, six replicates for each extract were made.

In tube dilution technique, to each 9 ml nutrient broth tube, 0.5 ml of different extracts (crude, alcoholic and phenolic) from various plant parts (vegetative leaf, reproductive leaf, vegetative stem, reproductive stem and rhizome) of *H. arifolia* were applied separately and 0.5 ml of three different inoculums were added in three different sets. The tubes were incubated in incubator shaker for different time period, up to 24 h at 37 °C, afterward; turbidity was measured at 600 nm in colorimeter. All experimental sets were done in six replicates for each.

Mycorrhiza and other root symbionts of *Hemionitis arifolia* were determined following Phillips and Haymann method (Phillips and Haymann 1970). Freshly collected root pieces of *H. arifolia* were thoroughly washed in tap water. And then were boiled for different duration (10 min, 20 min, 30 min, 40 min, 45 min and 50 min) in 10 % potassium hydroxide solution to standardize the digestion period. It was found that the roots of *H. arifolia* were digested perfectly in 45 min boiling period in the said condition. Properly digested roots were thoroughly washed in distilled water and then treated with 1 N hydrochloric acid for 1 min to remove trace of alkali. Then the roots were dipped in 0.05 % trypan blue stain for 24 h. After 24 h incubation excess stain was removed and roots were taken on a slide. Slide was prepared by spreading the thrashed root pieces evenly on grease free slide and then mounted with glycerol: acetic acid (1:1) solution for observation under various magnification of a compound microscope (LEICA DM1000).

Result

Extract from vegetative and reproductive leaves showed considerable inhibitory effect against Gram negative test strain (*Escherichia coli* MTCC-739) in both agar cup assay (Tables 1 and 2) and tube dilution assay (Fig. 1a, b) but neither leaf extract nor extracts from other parts of the plant showed inhibitory effect both in agar cup (Tables 3

Table 4 Agar cup assay of alcoholic extracts against *B. subtilis*

Samples	Diameter of zone of Inhibition (mm)
Vegetative Leaf	0
Reproductive leaf	10
Vegetative Stem	0
Reproductive Stem	0
Rhizome	0

and 4) and tube dilution assay (Fig. 1c, d) against Gram positive test strain (*Bacillus subtilis* MTCC-441) except the extract from reproductive leaves. It was observed that alcoholic extracts showed marginally better inhibitory effect over other extracts (Fig. 1, Tables 1 to 4). None of the extracts showed any antifungal activity against the test strain *Candida albicans* (MTCC-7353). Extracted phenol content of various plant parts was estimated (Table 5) but it did not show antimicrobial effect.

Various types of reproductive and vegetative structures such as zygosporangia, chlamydospores, vesicles, arbuscules, hyphal network and DSF of associated mycorrhizal members such as *Glomus mosseae*, *Glomus heterosporum* and DSF of the test plant was studied following Mycorrhiza (Prokash and Mahotra 2006) and Manual of Mycorrhizal research (Podila and Varma 2005) (Fig. 2a to f).

Discussion

From the obtained result it is evident that the pteridophyta species *Hemionitis arifolia* has considerable antibacterial activity, particularly against Gram negative bacterial test strain *Escherichia coli* MTCC-739 which may be enhanced many fold by adopting various minor modifications of the naturally present compounds. Alcoholic extracts showed more intense inhibitory effect than crude extracts and extract from reproductive leaves showed maximum antibacterial property. No antifungal activity was found in all type of extracts. It may be due to

Table 3 Agar cup assay of crude extracts against *B. subtilis*

Samples	Diameter of zone of Inhibition (mm)
Vegetative Leaf	0
Reproductive leaf	9
Vegetative Stem	0
Reproductive Stem	0
Rhizome	0

Table 5 Phenol concentration of various plant parts

Samples	Concentration of phenol (µg/ml)
Vegetative Leaf	550
Reproductive leaf	552
Vegetative Stem	120
Reproductive Stem	127
Rhizome	110

Fig. 2 Microphotograph of **a** Vesicle of VAM fungi, **b** Hyphal network of VAM fungi, **c** Zygospore of *G. mosseae*, **d** Chlamydospores of *G. heterosporum*, **e** Fungal hyphae of DSF, **f** Arbuscules of VAM fungi

the fact that root system of this plant has large number of symbiotically associated mycorrhizal and DSF strains. Types of vegetative and reproductive structures of mycorrhiza and DSF found in root system are amazing and may be studied further for their relation, if any, with antimicrobial activity of the host plant.

Acknowledgements The authors wish to thank Prof R.N. Mukhopadhyay, Dept. of Botany, Burdwan University, for identifying the Pteridophyta specimen. Thanks are also due to Dr. Sikha Dutta, Lecturer, Department of Botany, Burdwan University for identification of the mycorrhizal members. The bacterial cultures were gifted by Dr. Tapan Chakraborty while the yeast culture was gifted by Dr. G.S.Prasad, MTCC Chanidigarh-160036. We would also like to thank Department of Biotechnology, B.U. for their kind help.

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