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Characterization of antimicrobial compounds from a common fern, *Pteris biaurita*

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Methanol extract was prepared from the fronds of *Pteris biaurita* and partial purification was done by solvent partitioning with diethyl ether and ethyl acetate, followed by hydrolysis and further partitioning with ethyl acetate. The three fractions, thus obtained were bioassayed separately against five test fungi- *Curvularia lunata*, *Fomes lamaoensis*, *Poria hypobrumea*, *Fuasrium oxysporum* and a bacterium- *Bacillus pumilus*, by spore germination, radial growth and agar cup techniques. Results revealed that ethyl acetate fraction (III) contained the active principle. TLC plate bioassay of the active fraction revealed inhibition zone at an R_f of 0.5-0.65. Silica gel from this region was scraped, eluted in methanol and subjected to UV-spectrophotometric analysis. An absorption maxima of 278 nm was recorded. HPLC analysis of TLC-eluate revealed a single peak with retention time of 8.1 min. GC-MS analysis revealed six major peaks in the retention time range of 7.2-10.9 min. Comparison with GC-MS libraries revealed that the extracts may contain a mixture of eicosenes and heptadecanes.

Keywords: Antimicrobial compounds, Fern, HPLC, GC-MS

Pteris biaurita L., is a common fern that grows luxuriantly under varying habitats and is used for ornamental purposes. Its fronds are herbaceous with arching lamina. It is a very resilient plant and defends itself against microbial attack. There are reports of the medicinal properties of a few ferns, including *Polypodium* sp., *Adiantum* and *Selaginella* sp[1-3]. Ramashankar and Khare[4] reported about 58 species of ferns and fern allies from Pachmarhi, India, out of which about twelve were reported to have medicinal properties This created an interest and the present study was undertaken with a view to partially purify and characterize the antimicrobial compound(s) from this fern, if any, which in turn may prove useful for medicinal purposes.

Materials and Methods

Plant material—Plants of *Pteris biaurita*, growing wild in the University campus was collected and used. This was identified at the NBU Herbarium.

Test organisms—Five fungal cultures, *Curvularia lunata*, *Fomes lamaoensis*, *Poria hypobrumea*, *Fusarium oxysporum* and *Aspergillus niger* and a bacterial culture of *Bacillus* sp., were obtained from the culture collection of Immuno-Phytopathology Laboratory, Department of Botany, University of North Bengal. Four of the five fungi were selected for bioassay as these are common plant pathogenic fungi, while *A.niger* was selected being one of the most common fungi in the aerospora, as well as extremely fast growing. These were maintained in PDA and NA, respectively, and used for bioassay purposes.

Preparation of extract and solvent partitioning—Methanol extracts were prepared following the method of Daayf *et al*[5]. Leaf sample (50 g) was homogenized in 80% methanol (10 ml^{-1}g tissue), and was extracted on a rotary shaker in an Ehrlenmeyer flask at 40 rpm overnight. Extract was then collected by filtration using Whatman filter paper (No.9) and concentrated to a final volume of 20 ml. This was partitioned against equal volumes of anhydrous diethyl ether (Fraction I) and ethyl acetate (Fraction II) for 1h each. The aqueous fraction was hydrolysed with 4N HCl by boiling for about 1h. Hydrolysates were further extracted with ethyl acetate for 1h , concentrated and termed as Fraction III. All the fractions were brought to 1 ml. and used for bioassay. Chemicals used in the study were from E.Merck (Germany).

Bioassay

The different fractions were bioassayed by spore germination, radial growth, agar cup bioassay and TLC plate methods against the fungi and the bacterium.

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Spore germination assay—Fungal spores of the test fungi were bioassayed against the extracts on glass slides following the method of Trivedi and Sinha[6]. The extracts were spotted on the glass slides, allowed to dry and then the spore suspensions were placed on top of these. In case of control, ethyl acetate was first spotted on the glass slides, dried and then the spores were placed. At the end of 24h of germination, the spores were stained with lactophenol cotton blue, fixed, observed under the microscope and percentage germination was calculated. For each case, a total of about 500 spores were counted from about 5-8 microscopic fields.

Radial growth assay—The method of Fiddaman and Rossall[7] was followed with modifications. Autoclaved PDA medium (20 ml) was mixed with 0.5 ml of extract and poured into Petri plates (9 cm diam). After solidification, an agar block containing growing mycelium of the test fungus was placed in the center and allowed to grow. Control plates were mixed with solvent alone. Radial growth was measured after definite time intervals ie., after 2, 4, 6 and 8 days.

Agar cup assay—Antibacterial and antifungal activity was determined by agar cup bioassay technique. In antibacterial tests, an aqueous suspension of 24 h old *Bacillus* sp was made in sterile distilled water, from which, a loopful of the culture was spread evenly on solidified nutrient agar[8,9]. A well was made in the center of the plate with a cork borer and 200 μl of the extract or ethyl acetate (control) were placed in the wells. Plates were incubated at 37°C for 24 h after which the diameter of inhibition zone was noted. In antifungal tests, initially, wells were made in the periphery and extracts added as mentioned earlier. Following this, an agar block containing growing mycelium of the test fungus was placed in the center of solidified PDA medium. Growth was observed for 6 days after which the inhibition zones were noted.

Thin layer chromatography—For TLC plate bioassay, thin layer chromatography (TLC) was done on Silica Gel G (Himedia) coated on clean, grease-free slides, which were air dried and activated at 80-100° C prior to loading of samples. Aliquots (20μl) from the fraction showing antimicrobial activity in the other tests were spotted on the silica gel plates, and developed in ethyl acetate: benzene (9:11) solvent mixture as described by Chakraborty and Saha[10]. After the solvent had run upto 15 cm, chromatogram inhibition assay was performed as devised by Hofmans and Fuchs[11], using *Curvularia lunata* as the test organism. TLC plates were air dried and spore suspension in 2% sucrose supplemented Richard's medium were sprayed on the developed TLC plate and incubated in sterile humid chamber at 25°C for 6 days. Fungitoxicity was ascertained by the appearance of inhibition zone, which was visualized as white zones surrounded by deep black background of mycelia. R_f (rate of flow) values were determined. For determination of antibacterial activity, after the development of the samples in the solvent system, the TLC plate was overlayered with 0.7% nutrient agar inoculated with *Bacillus* sp. and incubated in sterile humid chamber at 37° C for 3 days. Antibacterial effect was ascertained by the presence of inhibition zone at specific R_f value[12].

UV-spectrophotometric analysis—For UV-spectrophotometry, ten spots of the fraction III were first of all spotted on to silica gel plates (preparative TLC), and were developed in the same solvent system. The silica gel from the required zone (corresponding to the zone showing antimicrobial activity) in preparative TLC was scraped off, eluted in methanol (spectrophotometric grade) and centrifuged at 10,000 rpm. The clear supernatant was then dissolved in 1 ml of methanol (spectrophotometric grade) and analysed by UV-spectrophotometry in a Beckman Spectrophotometer.

HPLC analysis—The eluate from TLC was also analysed by HPLC (Shimadzu, Advanced VP Binary Series) using a C-18 Hypersil (ODS) column. The eluate (20 μl) was injected into the column. Column was run using a binary gradient solvent of methanol-water initially-20% methanol, with an increase in concentration of methanol gradually upto 100% over a period of 30 min at a flow rate of 1 ml/min and analysed at 278 nm. Program was terminated at the end of 30 min.

GC-MS analysis—Analysis was carried out on a Shimadzu QP5050A model, EI mode, using a DB5 column (0.25mm OD × 30 meter). Flow rate was 1.5ml/min, solvent, hexane and programmed as 80°C (2min)–260°C at 40°C/min and held at 260°C for 25 min.

Results

Bioassays

Spore germination assay—All three fractions were tested against the test fungi to determine the antifungal activity. In each case, germination of 500

spores were counted. Results revealed that, among the three fractions, only Fraction III was inhibitory to spore germination of all the test fungi (Table 1).Spore germination was completely inhibited in *C. lunata* and *P. hypobrumea*.

Radial growth assay—Results of spore germination test was confirmed by radial growth bioassay. Radial growth of all the fungi were inhibited, ranging from 65-95% by Fraction III at the end of 8 days of growth (Table 2). Statistical analysis revealed that, growth in Fraction III was significantly lesser as compared to control, while the difference was not significant in the other two fractions.

Agar-cup assay—Antibacterial activity was exhibited by Fraction III of the extract, where a prominent inhibition zone was observed after 24 h of incubation (Fig.1A). No inhibition zones were observed in fractions I and II. All the test fungi also developed inhibition zones around the wells containing Fraction III of the extract (Fig.1 B, C). Mycelial growth of *F. oxysporum* was inhibited to a lesser degree by Fractions I & II (Fig. 1 B).

Table 1—Spore germination bioassay of methanolic extract from *P. biaurita* against different fungal species

[Values are mean ± SE of 500 spores]

Fungal species	% spore germination			
	Control[a]	Fr. I	Fr. II	Fr. III
C.lunata	99.0 ± 2.8	96.4±2.9	98.1±3.6	0.00
F.lamaoensis	98.5 ± 4.1	92.4±3.4	95.4±2.9	1.4±0.2
P.hypobrumea	98.0 ± 3.1	98.0±2.6	94.3±3.1	0.0
F.oxysporum	99.0 ± 2.3	97.3±1.8	96.2±1.1	2.8±0.5
A.niger	99.2± 2.4	97.4±2.7	95.8±2.5	5.6±0.7

[a]Ethyl acetate control

Table 2—Radial growth bioassay of methanolic extract from *P. biaurita* against different fungal species

[Values are mean ± SE of 3 replicates]

Fungal species	Diameter of radial growth (cm)			
	Control[a]	Fr. I	Fr. II	Fr. III
C.lunata	8.8±1.2	7.6±0.9	7.0±0.3	2.1±0.1
F.lamaoensis	9.0±1.0	7.8±0.4	7.6±0.9	1.4±0.2
P.hypobrumea	8.4±0.9	7.6±0.8	7.8±1.0	1.2±0.2
F.oxysporum	9.0±0.7	8.6±0.7	8.4±1.1	3.5±0.4
A.niger	9.0±1.3	8.5±1.0	8.7±0.9	2.9±0.3

[a]Ethyl acetate control

Difference in values of all tests between control and fraction III was significant at

P=0.01 using Student's t test. The rests were insignificant compared to controls.

TLC plate assay—Antifungal activity in Fraction III was further confirmed by TLC plate bioassay against one of the test fungi. Samples of Fraction III were loaded on TLC plates and bioassayed against *C. lunata* as described earlier. After a period of 6 days, observation revealed the presence of an inhibition zone only in Fraction III. Rf of this was calculated to be in the range of 0.5-0.65. Antibacterial activity was also obtained at the same Rf.

For further purification steps, the eluate prepared from the region showing antifungal/ antibacterial activity in a preparative TLC, was used.

UV-spectrophotometric analysis—Following pre-parative TLC and elution of the zone corresponding to the zone showing antimicrobial activity in TLC plate bioassay, UV-spectrophotometric analysis of the eluate was carried out in the range of 200-800 nm. A single peak with an absorbance value of 1.1 was obtained at 278 nm.

HPLC analysis—Since the absorption maxima in UV-spectrophotometric analysis was at 278 nm, UV-detection in HPLC analysis was done at 278 nm. Analysis by reverse-phase chromatography using methanol-water gradient, revealed the presence of a single peak, with a retention time of 8.1 min (Fig.2). Early elution of compound indicated the possibility of it being more hydrophobic in nature.

GC-MS analysis—Analysis of the eluate from TLC by GC-MS revealed six major peaks, with retention times of 7.2, 7.62, 8.08, 8.6, 9.22 and 14.14 min. (Fig. 3, Table 3). The peaks constituted 14.1, 14.0, 14.7, 11.3, 8.6 and 21.4% of total, respectively. This was compared to GC-MS libraries which revealed that these may be a mixture of eicosenes and heptadecanes.

Discussion

In the present study, *Pteris* , a commonly occurring fern of this region showed antimicrobial activity. Solvent extraction, fractionation and bioassays led to the isolation of a group of compounds showing strong antibacterial and antifungal properties, which was further characterized by UV spectrophotometry, HPLC and GC-MS In a previous study, twelve species of pteridophytes with medicinal properties were reported by Ramashankar and Khare[4]. Among pteridophytes, *Selaginella* is also well known for its medicinal properties[3,13-15]. Antiviral amentoflavone was isolated from *Selaginella sinensis* by Ma *et al.*[16]. Several workers have also reported the presence of

ptaquiloside and/or ptaquiloside like compounds in a number of ferns including *Pteridium esculentum*[17], *Dryopteris juxtaposita*[18] and species of *Cheilanthes*[19]. In the present study, characterization of the antimicrobial compound(s) by TLC, HPLC and GC-MS revealed active compounds might be a mixture of eicosenes and heptadecanes, though further analysis would be needed to confirm this. GC-MS analysis of chloroform extracts in flowers, stems and roots of *Tripleurospermum callosum* revealed the identification of 93 compounds[20]. Studies on chemical composition and antibacterial activity of the essential oil of *Centella*

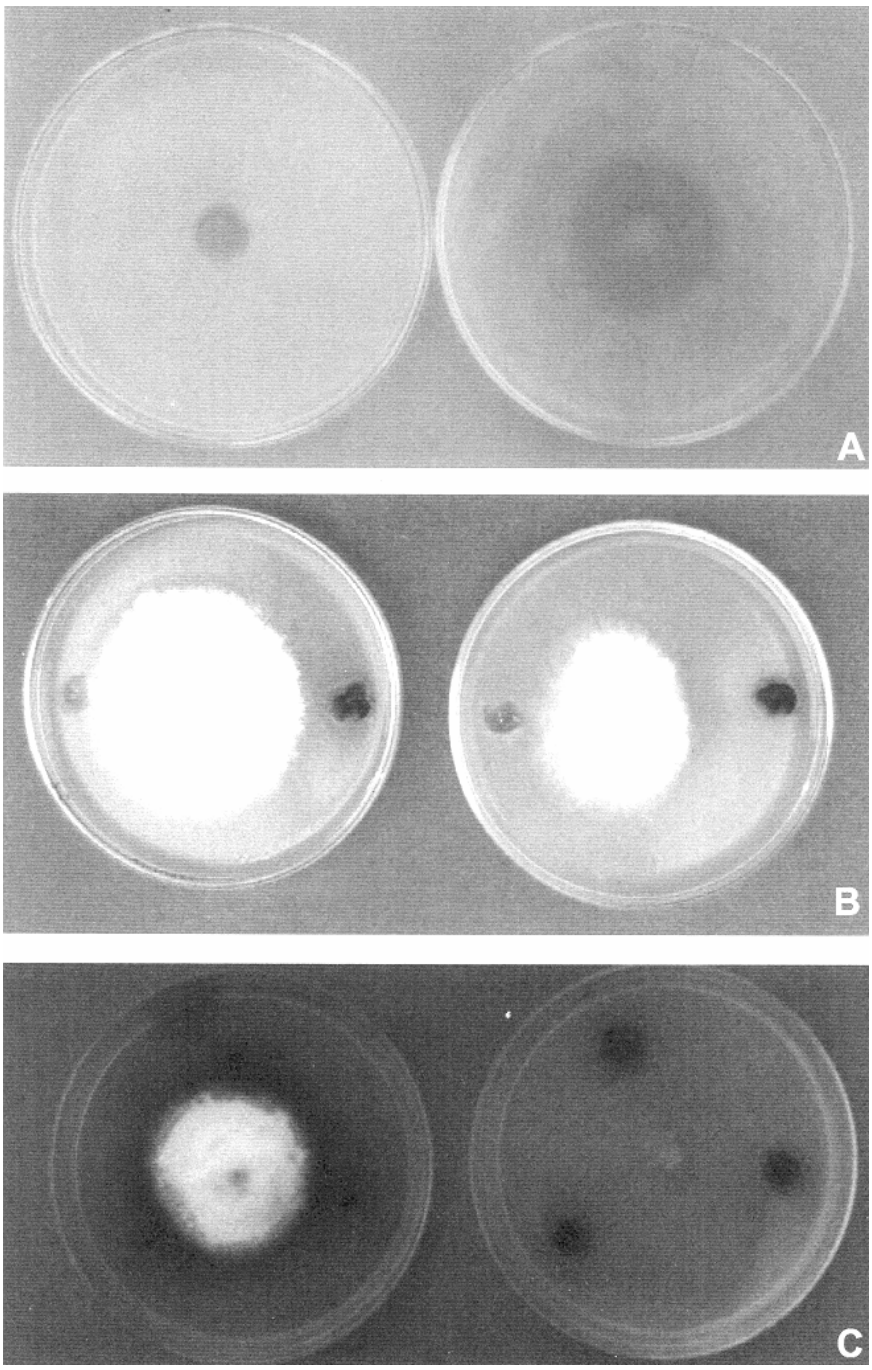

Fig. 1—Antifungal and antibacterial activities of *Pteris biaurita* extract. (A) Inhibition of growth of *Bacillus* sp. by Fraction III of extract (Left plate- control, Right plate with well containing extract); (B) Inhibition of mycelial growth of *Fusarium oxysporum* (Left plate, left well- control; Left plate right well- Fraction I; Right plate, left well-Fraction II; Right Plate, right well- Fraction III); and (C) Inhibition of growth of *Fomes lamaoensis* (Left plate-control; Right plate- all wells with Fraction III of extract).

Table 3—GC-MS analysis of TLC eluate from extract of *P.biaurita*

Peak No.	R. Time (min.)	I.Time (min.)	F.Time (min.)	Area	Height	A/H	% Total
1	7.195	7.158	7.250	2002146	1149704	1.74	14.11
2	7.616	7.583	7.633	1992898	1913141	1.04	14.04
3	7.650	7.633	7.692	1059550	863312	1.22	7.47
4	8.076	8.050	8.117	2088025	1973771	1.05	14.71
5	8.600	8.575	8.633	1610061	1348371	1.19	11.34
6	9.220	9.192	9.267	1220869	829497	1.47	8.60
7	9.972	9.942	10.008	732877	472832	1.54	5.16
8	10.901	10.858	10.950	454394	237129	1.91	3.20
9	14.139	14.075	14.225	3032647	946764	3.20	21.37

R.time = Retention time; I. time= Ionization time ; F.time= Fragmentation time

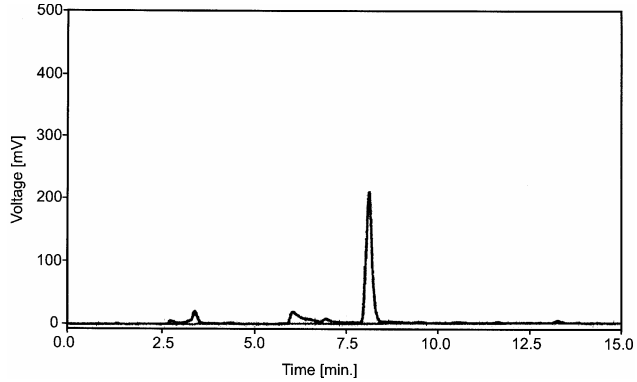

Fig. 2—HPLC analysis of antifungal component of *Pteris biaurita* extract following TLC run and elution from TLC plate.

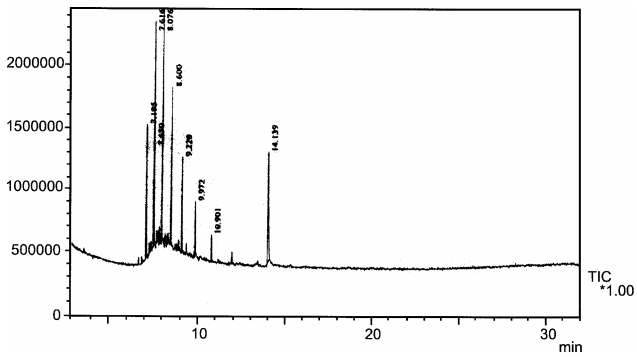

Fig. 3—GC-MS analysis of antifungal component of *Pteris biaurita* extract following TLC run and elution from TLC plate.

asiatica from South Africa was conducted by Oyedeji and Afolayan[21], who have reported that a number of hydrocarbons are present in *C.asiatica*. Bonsignore et al.[22] isolated some antimicrobial terpenes, one of which was identified as 8,10,heptadecadiene- 4,6 divnel-12 diol from *Oenanthera crocata*, which was effective against *Streptococcus facealis* and *Bacillus leutus*. Ozdemir *et al*.[23] have reported that the volatile, antimicrobial component of *Spirulina platensis* consisted of hepta and tetradecanes as major components.

The present study, therefore, indicated that *Pteris biaurita* had strong antimicrobial activity, which might be due to the eicosenes and heptadecanes. This plant has the potential to be utilized as a medicinal plant.

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