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Antimicrobial activity of leaf extract of *Basilicum polystachyon* (L) Moench

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Phenolic extract of leaves of *Basilicum polystachyon* (L) Moench was tested for *in vitro* antimicrobial activity against five bacteria (*Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Bacillus subtilis*, *Micrococcus leuteus*) and three fungi (*Fusarium oxysporum*, *Aspergillus niger*, *Helminthosporium oryzae*). Efficacy of organic solvents, methanol and ethanol, as agents for extraction was compared with acidic water (2*M*; HCl). High-pressure liquid chromatographic (HPLC) data showed that acidic extraction (2*M*; HCl) resulted in higher yield of caffeic acid (0.437 mg g^{-1}) and rosmarinic acid (0.919 mg g^{-1}). Acidic extract showed high activity against Gram (+) ve bacteria, but was less active against Gram (-) ve bacteria. Amongst the tested fungi, maximum activity was exhibited against *Aspergillus niger*. This is the first report on the phenolic constituents and bioactivity of *B. polystachyon*.

Keywords: Antimicrobial activity, *Basilicum polystachyon*, Caffeic acid, Rosmarinic acid

Basilicum polystachyon (L) Moench of Lamiaceae is an important ethno-medicinal plant growing in moist shady places during the monsoons (July-December) in India[1]. Plants belonging to the family Lamiaceae contain a large number of polyphenolic compounds[2] that are involved in a wide range of physiological and ecological activities providing resistance to bacterial, fungal and viral infections[3]. Among the various polyphenolic compounds in the mint family having antioxidant properties, rosmarinic acid is the most abundant caffeic acid ester[4].

The first step in the process of obtaining secondary metabolites from biogenic materials is to release them from the matrix by means of extraction[5]. As there are no available reports on the polyphenol content of *B. polystachyon*, the extraction procedure has to be standardized. Many workers use organic solvents like methanol or ethanol for the extraction of phenolic compounds[6]. Keeping in view the current trend of using herbal extracts as neutraceuticals and pharmaceuticals, the present study has been undertaken to investigate the antimicrobial activity of caffeic acid and rosmarinic acid from *B. polystachyon*.

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Materials and Methods

Plant material—Plant materials were collected from Chot Balon in Burdwan, India, identified and the voucher specimens submitted at The Burdwan University Herbarium (BURD) of the department.

Chemicals—Caffeic acid and rosmarinic acid as standards (Fluka Chemi GmBH) and HPLC-grade solvents (SRL, India) were used for chromatography. All the other chemicals were of analytical grade. Milli-Q water was used for extraction and HPLC.

Extraction of plant material—Freshly harvested leaves were used for analysis. Total phenolics were determined with Folin-Ciocalteau reagent[7], which takes into account all hydroxylated aromatic compounds and expressed as gallic acid equivalents (mg GA g^{-1} fresh wt). Extraction for phenolic acids was done following the method of Harborne[6]. Leaves (10 g) were cut into small pieces and extracted with 50 ml of HCl (2*M*) for 30 min in a boiling water bath. The resultant solution was partitioned with ethyl acetate in a separating funnel (x 3), the organic phase was concentrated in vacuum, dissolved in 50% HPLC-grade methanol and kept at 4°C for further study.

For comparative analysis of the effect of organic solvents on extraction process, 200 mg leaves were crushed in 10 ml of 80% methanol (MeOH) or 70%

ethanol (EtOH), centrifuged (5000 rpm) and the resultant supernatant pooled and concentrated. For analysis of the effect of acid treatment[8], an aliquot of the methanolic extract was acidified (2*M*; HCl), refluxed for 30 min in boiling water bath, cooled, partitioned with ethyl acetate, evaporated in vacuum, dissolved in 50% HPLC-grade methanol and kept at 4°C until further analysis. Ester bound phenols incorporated in the cell wall were extracted after alkaline (NaOH) hydrolysis[9].

HPLC analysis of caffeic acid and rosmarinic acid—For quantitative estimation of caffeic acid and rosmarinic acid, HPLC was performed using a RP C18 Techsphere (UK) 50 ODS column (25 cm × 4.6 mm) on a Waters BREEZE^{TM} system (Waters, Milford, CA, USA). The polar mobile phase comprised with methanol and aqueous 1 m*M* triflouroacetic acid (40:60) at a flow rate 1 ml min^{-1} for 15 min with detection at 330 nm. To minimize variation in quantification, samples were taken in triplicate.

Test organisms—Tested bacterial strains (*Escherichia coli*, *Pseudomonas aeruginosa, Staphylococcus aureus*, *Bacillus subtilis*, *Micrococcus leuteus*) were obtained from the Dept of Microbiology, Vidyasagar University, India and the fungal strains (*Aspergillus niger*, *Fusarium oxysporum*, *Helminthosporium oryzae*) were obtained from Mycology and Plant Pathology Laboratory, Botany Dept, Burdwan University, Burdwan, India.

Antimicrobial activity—The major detected phenolic constituents in the acidic (2*M*; HCl) leaf extract were caffeic acid (11.75%) and rosmarinic acid (24.77%). The acidic extract was partitioned with ethyl acetate, evaporated, residue dissolved in 50% MeOH, lypolysed and kept at 4°C. Antibacterial activity of the lypolysed extract was tested using both filter disc and broth dilution method following Bais *et al*[3]. An aliquot of individual bacterial stock was grown overnight at 30°C in 5 ml liquid LB medium[10] for inoculation. Inoculum (50 µl) was added to culture medium supplemented with various concentrations (50, 100, 150, 200, 250 µg ml^{-1}) of extract initially dissolved in DMSO. Individual bacterial culture without extract was used as control. Bacterial growth was measured at 540 nm after 24 h. For disc diffusion assay, 20 µl individual bacterial cultures was poured with LB agar medium (30 ml) in petri plates (90 mm). Filter discs (5 mm) were soaked with extracts at concentrations of 50-250 µg ml^{-1} and placed on petri plate in circular manner. Inhibition zone was measured after 24 h incubation at 30°C.

Antifungal assays were performed based on the method of Hsu *et al*[11] with slight modifications. Extracts dissolved in DMSO were added to PDA medium in perti plates (90 mm) to a final concentration of 50-250 µg ml^{-1} and after transferring the mycelia of fungal strains, the dishes were incubated in dark at 26°±2°C and 70% relative humidity. When the fungal mycelia reached the edges of the control dishes, the antifungal indices were calculated:

$$\text{Antifungal index (AI \%)} = 1 - (D_a / D_b) \times 100$$

where D_a is the diameter of growth zone in the experimental dish (cm) and D_b is the diameter of growth zone in the control dish (cm). Each test was repeated three times and the average was calculated. The IC_{50} and IC_{90} values were calculated by probit analysis (STATSDIRECT version 2.5.7, 2006).

Statistical analysis—The significance of difference was calculated using Tukey HSD test (VassarStats © Richard Lowry 1999-2007, http://faculty.vassar.edu/lowry/webtext.html) and results with $P < 0.05$ were considered to be statistically significant. All results were expressed as mean ± standard error. Experiments were performed in triplicates, each containing five treatments and a control.

Results and Discussion

Both methanolic and ethanolic solvents gave lower yields of caffeic acid and rosmarinic acid compared to acidic water (2*M*; HCl) used as the extraction solvent (Fig. 1). Treatment of methanolic extract with HCl resulted in extraction of considerable amount of phenolic acids. But, HCl and NaOH treatment of the residue after methanolic extraction yielded little amount of the caffeic acid and rosmarinic acid. Standard caffeic acid and rosmarinic acid was found to be stable in 2 *M* of HCl over a 3 h period indicating that initial mild acid extraction of the leaves for 30 min did not have any deleterious effect on the phenolic concentration. Total phenolic content of the acidic extract was 3.7 mg GA g^{-1} fresh wt. Following HPLC analysis (Fig. 2), caffeic acid and rosmarinic acid content was found to be 0.436 and 0.919 mg g^{-1} fresh wt, respectively. The HPLC elutes were checked by UV-vis spectrum overlay with caffeic and rosmarinic acid standards.

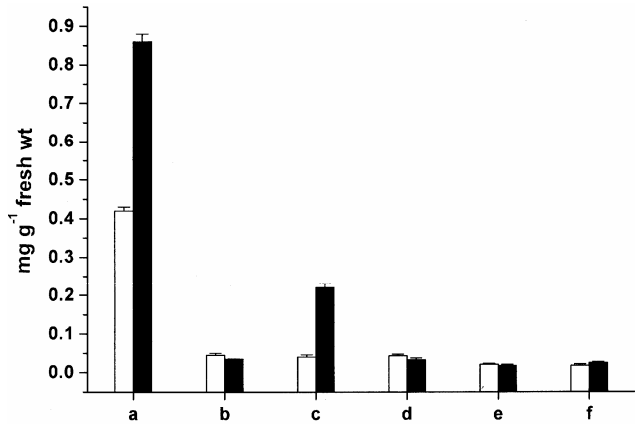

Fig. 1—Efficacy of various solvents on the yield of caffeic acid (□) and rosmarinic acid (■) from the leaves of *B. polystachyon*. [(a) HCl extract; (b) 80% MeOH extract; (c) 80% MeOH extract + HCl; (d) MeOH residue + NaOH; (e) MeOH residue + HCl and (f) 70% EtOH extract].

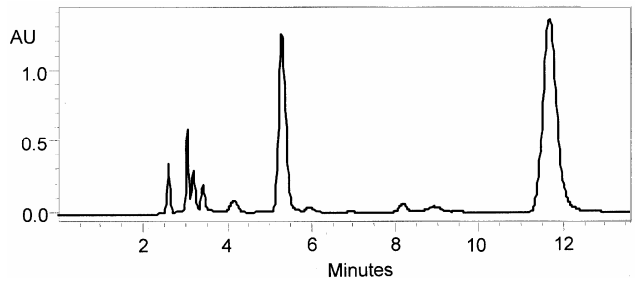

Fig. 2—HPLC chromatogram of *B. polystachyon* leaf extract at 330 nm. Caffeic acid (Rt: 5.3 min), rosmarinic acid. (Rt: 11.87 min).

Alcohols (methanol, ethanol) are frequently used to extract phenolic compounds from plant material[6,12]. Different extraction solvents, such as petroleum ether, chloroform, ethyl ether, ethyl acetate, and acetone have been tested for extraction steps involving removal of interfering compounds. In *B. polystachyon*, treatment of the methanolic extract with HCl resulted in increased release phenolic acids as compared to initial methanolic extract indicating the presence of glycosidic bonds. Rosmarinic acid-caffeic acid conjugates are also reported to be present in Lamiaceous plants[4]. Several simple phenylpropanoids (with basic C6-C3 carbon skeleton of phenylalanine) are free acids and rarely accumulate to high levels inside the plant cells, instead they are usually conjugated to sugars, cell wall carbohydrates or organic acids[8,13]. Presence of such complex components might explain the increased amount of phenolic acids post-acid treatment. In *B. polystachyon*, caffeic and rosmarinic acids are not conjugated to cell wall carbohydrates in high amounts, as saponification with NaOH or hydrolysis of the cell wall residue with HCl did not increase the yield. Extraction by HCl (2 *M*) is therefore, beneficial, as it breaksdown the glycosidic bonds present, if any, and at the same time solubilizes the polyphenolic compounds which are not extracted on using alcohols as solvent. Previous reports on Lamiaceous plants show contrary results of acid treatment after an initial methanolic extraction, with a decrease in rosmarinic acid content in basil, orgeno, savory and thyme extracts, but increased content in rosemary and sage extracts[8]. It is interesting to note that all of these plants contain high amounts of rosmarinic acid. Thus, extraction procedures are plant specific and a compound may not form the same kind of conjugate in different species. Water extracts obtained from herbs of Lamiaceae have been reported to be rich in phenolic compounds such as hydroxycinnamic acids and flavonoids[14]. A mild acidic extraction followed by ethyl acetate partitioning is reported to increase antimicrobial activity of a number of medicinal plants in comparison to organic solvents and has been recommended for testing bioactivity[15]. In the present study with *B. polystachyon*, acidic extraction followed by an ethyl acetate purification step was adapted to isolate phenolic acids and test their bioactivity.

The leaf extract of *B. polystachyon* showed significant inhibitory activity against the tested bacterial (Fig. 3) and fungal strains (Table 1). The antimicrobial activity was measured by their growth (OD at 540 nm) with respect to control. Highest activity was observed against *M. leuteus* and *B. subtilis* among the tested bacterial strains. *E. coli* was least inhibited followed by *P. aeruginosa* and *S. aureus*. The antimicrobial activity was checked using disc diffusion assay and visually recorded after 24 h incubation (data not shown) which indicated similar results as the broth assay. The extract showed highest antifungal activity against *A. niger,* where the index was 71.6%. At 250 µg ml^{-1} extract concentration, *F. oxysporum* and *H. oryzae* exhibited antifungal indices of 59.9 and 63.9%, respectively (Table 1). IC_{50} and IC_{90} values (Fig. 4) were found to be highest in *F. oxysporum* among the tested fungi. IC_{90} values were 1.6 times higher than IC_{50} values for both *A. niger* and *H. oryzae* but it was 1.8 times higher in *F. oxysporum* indicating the former two to

Table 1—Antifungal Index (%) of different concentrations of leaf extract of *B. polystachyon*
[Values are mean ± SE of 3 replications]

Fungi	Phenolic extract (µg ml^{-1}) of *B. polystachyon*.				
	50	100	150	200	250
A. niger	1.1 ± 0.9*	18.4 ± 4.1	41.3 ± 3.5	54.2 ± 3.2	71.6 ± 1.7
F. oxysporum	10 ± 2.9*	21.6 ± 2.9	33.9 ± 2.8	47.96 ± 2.7	59.9 ± 2.7
H. oryzae	0.7 ± 0.5*	16.2 ± 2.5	35 ± 2.2	51.8 ± 2.2	63.9 ± 1.7

Values followed by * are not significantly different from the control at $P < 0.05$.

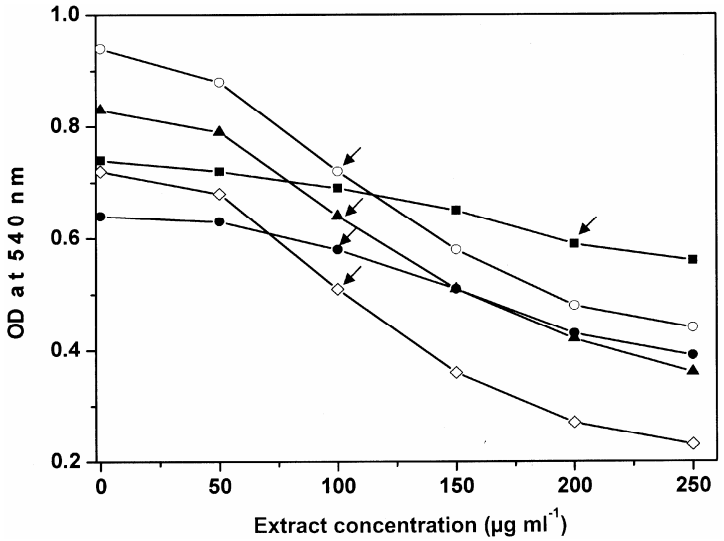

Fig. 3—Antibacterial activity of phenolic extract of *B. polystachyon* on five bacterial strains: *Escherichia coli* (■), *Pseudomonas aeruginosa* (●), *Staphylococcus aureus* (▲), *Bacillus subtilis* (○), *Micrococcus leuteus* (◊). Concentrations at and above those marked by arrows are significant at $P < 0.05$ for each of the tested bacterial strains.

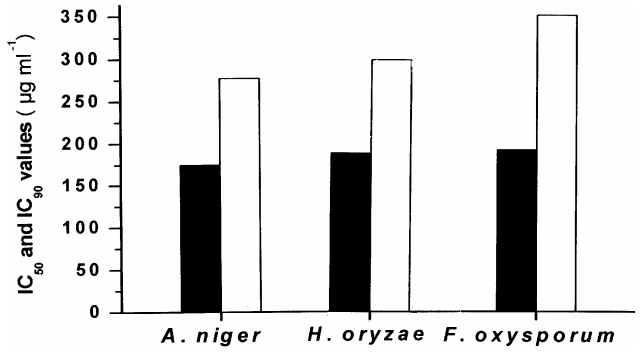

Fig. 4—IC_{50} (■) and IC_{90} (□) values of leaf extracts of *B. polystachyon* against tested fungi.

be more sensitive to the extracts than *F. oxysporum*.

In vitro antimicrobial activity of the leaf extract of *B. polystachyon* against the five bacterial and three fungal strains, might be attributed to the presence of high levels of polyphenolic compounds, caffeic acid and rosmarinic acid. Currently, microbial resistance to antibiotics has become a global concern as it spans nearly all known classes of natural and synthetic compounds fueling the need of new principles for antibacterial therapy[16-18]. New classes of drugs are, therefore, being synthesized that work on different target sites to those in current use and plants provide a ready source of such principles. It has been hypothesized that phenolic compounds play a role of phytoanticipins in plants[19]. Earlier workers[3,20] have observed that caffeic acid and rosmarinic acid have strong antibacterial property against Gram (+) bacteria and partial inhibition of Gram (-) bacteria. The Gram (-) bacteria differ from Gram (+) bacteria in having a thick liposaccharide coated cell wall which might be impermeable to the polar phenolic acids. It has been postulated that the antibacterial properties of rosmarinic acid is due to nucleoid damage with an increase in spatial division and condensation of genetic material[3]. The antifungal

activity of polyphenolic compounds might be due to the formation of multinucleate stage by the breakage of intersepta in mycelium and cell surface damage by pilferage[3]. Pharmacological, pharmaceutical botany, medical and clinical microbiology, phytopathology, and food processing are some fields in which phenolic compounds can be applied. *Basilicum polystachyon* showed potential evidence for its ethnopharmocological use and promising broad-spectrum antimicrobial drug.

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