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Antimicrobial activity of *Pavetta indica* leaves

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ABSTRACT

Antimicrobial activity of the aqueous and organic solvent extracts of the leaves of *Pavetta indica* were tested against *Bacillus subtilis*, *Escherichia coli* and *Saccharomyces cerevisiae* using disc diffusion assay. Most of the leaf extracts showed bactericidal activity against *B. subtilis*. None of the extracts exhibited any activity against *E. coli* and *S. cerevisiae*. Minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), thermal stability and qualitative phytochemicals studies were performed. Both MIC and MBC of the aqueous and methanol extracts were found to be between 1.95 - 7.81 mg/ml. The activity of aqueous and methanol extracts were found to be stable despite thermal treatment. Phytochemical analysis of aqueous extract revealed the presence of flavonoids, saponins and carbohydrates. Methanol extract was found to be positive for saponin and cardiac glycosides. TLC and bioautography were also done to identify the active fractions responsible for the antimicrobial activities. Results showed the presence of a number of bactericidal components. The study suggests *P. indica* to be a source for isolation of antibacterial compounds for human health care and use as preservatives in food processing industry.

INTRODUCTION

Pavetta indica Linn. belongs to the family Rubiaceae. It is widely distributed from the Andaman Islands, India and the north-western Himalayas to southern China and southwards throughout Malaysia to northern Australia. *P. indica* is a shrub or small tree of 3-5 m in height, with opposite branches having leaves that are membranous and variable in shapes and sizes. *P. indica* leaves are used in the treatment of liver disease, pain from piles, urinary diseases and fever (Kirtikar and Basu, 1975; Thabrew *et al*., 1987). It is a medicinally important plant having anti-inflammatory activities (Mandal *et al*., 2003). Golwala *et al*. (2009) reported analgesic activity of leaf extract of *P. indica*. Its root extract also have diuretic and purgative activity (Kumar, 2006). Other species of *Pavetta* also showed different biological activities. *P. gardeniifolia*, *P. pyroides* and *P. crassipes* have been reported to possess anti-malarial activity (Sandra *et al*., 2009; Gbeassory *et al*., 1989). Schistosomicidal properties of ethanol and acetone extracts of *P. owariensis* have been reported by Balde *et al*., (1989).

Amos *et al*. (2004) have shown that the extracts of *P. crassipes* dose-dependently decreased spontaneous motor activity (SMA) in mice and attenuated amphetamine-induced hyperactivity and the different episodes of stereotypic behavioral patterns induced by amphetamine.

The antimicrobial activities have been very briefly mentioned in some other species of *Pavetta* (Balde *et al*., 1990; Balde *et al*., 2010; Anago *et al*., 2011) but to our knowledge it has not been characterized adequately to assess its potential as future therapeutic source for combating microbes. In this report we describe preliminary studies to show the existence of bactericidal (Gram-positive) and bacteriostatic (Gram-negative) activities in the leaf extracts of *Pavetta indica* Linn. for the first time. The phytochemicals produced by the plants for their self protection have been demonstrated to protect human against a number of diseases. Antimicrobial activities of phytoconstituents such as phenol, tannins, terpenoids, essential oils, alkaloids, saponins and flavonoids have been reported by several authors (Weimann *et al*., 1997; Atindehou *et al*., 2002; Edeoga *et al*., 2005; Ahmed *et al*., 2012; Kamal *et al*., 2010) in other plant systems. We have done preliminary experiments to obtain the phytochemical profiles in leaf extracts of this species for correlating the observed antibacterial activities with these phytoconstituents.

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MATERIALS AND METHODS

Collection and identification of plant materials

Fresh leaves of *P. indica* were collected from Santiniketan, West Bengal, India. The plant *Pavetta indica* Linn., was identified by Prof. K. N. Bhattacharya, Botany Department, Visva-Bharati University, India and the dried specimen was deposited in the Herbarium of the Botany Department, Visva-Bharati University, India.

Preparation of plant extracts

Fresh leaves of *P. indica* were air dried under shade and then ground into fine powder with the help of an electric grinder. For extraction of the dried powder, aqueous and organic solvents i.e., methanol, chloroform, benzene and hexane were used. Air-dried powder of *P. indica* leaves (10 g) in solvents (1:10, w/v) was taken in a 250 ml conical flask and kept at room temperature for 5 days. After this period, the samples were filtered, supernatants were collected and solvents were evaporated to dryness. This process was repeated two more times and final dry extracts were stored at 4 °C until use.

Growth and maintenance of microorganisms

The dried aqueous and solvent extracts dissolved in dimethylsulphoxide (DMSO) were screened against *Bacillus subtilis* (MTCC 121), *Escherichia coli* (MTCC 484) and *Saccharomyces cerevisiae*. Stock cultures of *B. subtilis* and *E. coli* were maintained on slants or plates of LB (Luria-Bertani)-agar whereas *S. cerevisiae* on YPD (Yeast extract powder-Mycological peptone-Dextrose)-agar at 4 ℃. Inoculums of healthy cultures for experiments were prepared by transferring a single colony from the stock culture plate to 1.5 ml of LB broth or YPD broth and incubated at 180 rpm for 18 h at 30 ℃ (for *B. subtilis* and *S. cerevisiae*) or at 37 °C (for *E. coli*). Appropriate quantities of these inoculums were then directly used for larger liquid cultures or diluted suitably in 0.9% saline before use for antimicrobial screening. The general microbial techniques and compositions of LB, YPD broth and LB or YPD-agar are described by Sambrook and Russel (2001).

Antimicrobial activity screening

Screening of antimicrobial activities of the extracts were performed using the disc diffusion method (Bauer *et al*., 1966) and has already been described in Gupta *et al*. (2010). The plates were prepared by pouring 25 to 30 ml of media into sterile 90 mm Petri dishes. After adjusting the turbidity of the inoculums prepared according to the method described above, a sterile cotton swab was dipped into the suspension and was spread uniformly on agar plates. Then sterile Whatman no. 1 filter papers (6 mm diameter) were placed on the spread surface of the Petri dishes and 3 µl of dried extract (dissolved at 250 mg/ml in DMSO) was spotted on each of the filter papers. After this, the plates were then incubated at respective ambient temperatures for 18 h (for *B. subtilis* and *E. coli*) and upto 36 h (for *S. cerevisiae*). Subsequently, the zone of inhibition formed around the discs was measured. Ampicillin (250 µg/ml) and commercial Fluconazole (10 mg/ml) were used as positive controls for all such experiments.

Minimum inhibitory concentration (MIC) determination

MIC was performed by Guerin-Faublee *et al.* (1996) method and has been described by Gupta *et al*. (2010). It was done by carrying out the disc diffusion tests with discs spotted with serially diluted concentrations of aqueous and methanol extracts. For making serial dilutions, the dried crude extracts were dissolved in DMSO at a concentration of 250 mg/ml and serially diluted (1:1) with media to concentrations of 125, 62.5, 31.3, 15.6, 7.8, 3.9, 1.95 and 0.98 mg/ml for use. The lowest concentration of a particular extract that inhibited the growth of *B. subtilis* was noted as the MIC value of that extract for the *B. subtilis*.

Minimum Bactericidal Concentration (MBC) determination

The method of Greenwood (1989) was used to determine the MBC of the aqueous and methanol extracts. Serial dilutions of these crude extracts were made with sterile LB broth in test tubes in the range of 15.64, 7.82, 3.91, 1.95, 0.98 and 0.49 mg/ml. Then overnight grown test organism (10 µl) was pipetted into each of these test tubes containing various concentrations of the crude extracts as mentioned above and incubated at 30 °C for 24 h. At the end of this incubation period, the contents of the tubes were plated on LB-agar and grown for 18-20 h at 30 °C to determine the bactericidal activities in extracts. The concentration of crude extract at which no microbial growth occurred (as evidenced from absence of subsequent microbial growth in the LB-agar) was recorded as the MBC of that crude extract.

Thermal stability test

To determine the effect of temperature on the stability of leaf extracts, these extracts in 1.5 ml microfuge tubes, each with 250 mg/ml concentration of the crude extracts in DMSO, were treated at 40, 60, 80, and 100 °C and autoclaved at 15 psi, all for 15 min, separately. The samples were cooled to room temperature and the residual antibacterial activities were determined against the target organisms with the help of disc diffusion method described above.

Preliminary phytochemical studies

Qualitative phytochemical tests of *P. indica* leaf extracts were carried out for detecting the presence of saponins (by foaming test), carbohydrates (by Fehling's test), cardiac glycosides, flavonoids etc. and were detected using colour development tests as described by Harborne (1998) and Khandelwal (2000).

Thin Layer Chromatography (TLC)

TLC silica gel 60 F254 plates (Merck), 20 X 20 cm^2 and 0.2 mm thick, were used for TLC. Water and methanol extracts of leaf at a concentration of 50 mg/ml (6 µl; dissolved in methanol) were applied on TLC plates. The chromatograms (in duplicate)

were run using benzene: ethanol: ammonia (BEA, 18:2:0.2) or ethyl acetate: methanol: water (EMW, 10:1.35:1) as running solvents. At the end of the run, the TLC plates were subjected to bioautography as detailed below.

Bioautography

Bioautography technique of Nostro *et al*. (2000) was used with some modifications for the detection of antimicrobial components present in the leaf extracts, after separating these components on TLC plates as described above. At the end of TLC run, 1% inoculums of *B. subtilis* or *E. coli* containing LB-agar were poured on TLC plates. After solidification of the LB-agar medium on the TLC plates, they were incubated for 24 h at 30 °C and 37 °C for *B. subtilis* and *E. coli* respectively. Subsequently, the bioautogram was sprayed with a 1% aqueous solution of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) based on the reduction of MTT by mitochondrial dehydrogenase of viable cells resulting in a blue formazan product after incubation. It was then incubated at 30 °C for few minutes. Zones in which bacterial growth was inhibited failed to take the bluish stain and indicated the presence of active compounds in that area of TLC plate.

RESULTS

Disc diffusion tests of water and organic (methanol, chloroform and benzene) extracts of *P. indica* leaves showed appreciable antimicrobial activity against *B. subtilis*, the Gram-positive bacteria. But none of these extracts exhibited any growth inhibitory effects on *E. coli*, the Gram-negative bacteria or *S. cerevisiae*, the fungus (Table 1).

Table. 1: Antimicrobial activity of *P. indica* leaf extracts against test microorganisms by disc diffusion method.

Extracts/ controls	Zone of inhibition (mm)		
	B. subtilis	*E. coli*	*S. cerevisiae*
Aqueous	7.5	No	No
Methanol	7.5	No	No
Chloroform	6.5	No	No
Benzene	6.5	No	No
Hexane	No	No	No
DMSO	No	No	No
Ampicillin	28.0	13.0	-
Fluconazole	-	-	18.5

All the extracts used at 750 µg/disc. Inhibition zone diameter including disc diameter of 6 mm. No = No detectable inhibition. (-) = Not tested. DMSO was used as negative control. Ampicillin (250 µg/ml) and Fluconazole (10 mg/ml) were used as positive controls at 3 µl/disc.

Table. 2: MIC and MBC values of aqueous and methanolic extracts of *P. indica* leaf against *B. subtilis.*

Extracts	MIC (mg/ml)	MBC (mg/ml)
Aqueous	3.91 - 7.81	3.91 - 7.81
Methanol	1.95 – 3.91	1.95 - 3.91

MIC and MBC results of water and methanol extracts of leaves are shown in Table 2 which indicate that these extracts have bactericidal activities. Both MIC and MBC values ranged between 1.95-7.81 mg/ml. The thermal stability tests of the antibacterial activities found in aqueous and methanolic crude extracts were carried out by treating the extracts dissolved in DMSO at 40, 60, 80, 100 °C and by autoclaving (121 °C) for 15 min. The results obtained against *B. subtilis* have demonstrated very little or no loss in these activities in comparison to the untreated samples (Table 3). Phytochemical analyses revealed the presence of saponins, carbohydrates and flavonoids in aqueous extract and also saponins and cardiac glycosides in methanol extracts (Table 4).

Table. 3: Thermal stability test of *P. indica* leaf extracts against *B. subtilis.*

Temperature (°C)	Antimicrobial activity (mm)	
	Aqueous extract	Methanol extract
40	7.75	7.75
60	7.75	7.75
80	7.75	7.75
100	7.75	7.75
Autoclaved	6.75	7.75
DMSO	No	No

Extracts used at 750 µg/disc. Inhibition zone diameter including disc diameter of 6 mm. No = No detectable inhibition. (-) = Not tested. DMSO was used as negative control.

Table. 4: Phytochemical analysis of *P. indica* leaf extracts.

Phytochemicals	Aqueous extract	Methanol extract
Saponins	+++	+
Carbohydrates	+	-
Cardiac glycosides	-	+
Flavonoids	+++	-

+ = Present in low amounts; +++ = Present in high amounts; - = absent

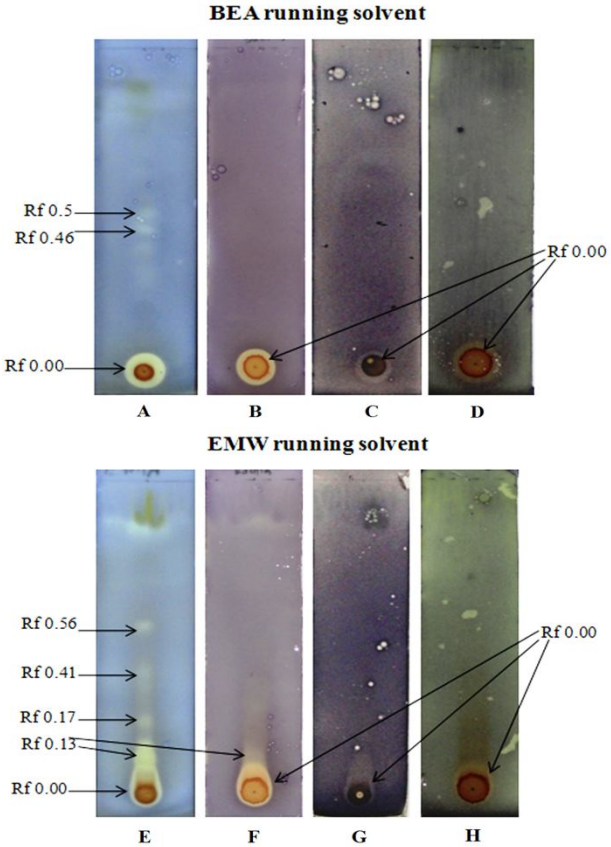

Fig. 1: TLC-bioautography of methanol and aqueous extract of leaf of *P. indica* in different running solvents. Lanes A, B, E and F = Bioautography against *B. subtilis*. Lanes C, D, G and H = Bioautography against *E. coli*. Lanes A, B, C and D were run under BEA and E, F, G and H were run under EMW running solvents system respectively. The R_f values of different components are shown in the margins.

TLC separation of different phytochemicals present in the leaf extracts of *P. indica* followed by bioautography using *B. subtilis* demonstrate that under polar running solvent system (EMW), the anti-gram positive bactericidal activity resolves into several areas on the TLC plate (shown by arrows in Fig. 1 lane E) where killing of the *B. subtilis* is clearly visible in the form of colourless spots; these spots are areas on TLC plates that failed to take MTT stain due to absence of live bacteria in there. When the bioautography was performed using *E. coli* for identification of the active fraction on the TLC plates, it was seen that the zones of inhibition are not as clear as in the case of *B. subtilis* but diffused which suggests that while the compounds that are present in these spots are not able to kill *E. coli* (i.e. are not bactericidal for *E. coli*), they certainly are acting as growth inhibitors against *E. coli* (shown by arrows in Fig. 1 lanes C, D, G and H). It is also to be noted that these spots did not resolve well (figure 1 lanes A, B, C and D) when TLC was carried out in non-polar solvent system (BEA).

DISCUSSION

Genus *Pavetta* has about 200 species (Mouly *et al*., 2009); among them *P. indica* is known to be used in ethnomedicine for the treatment of microbial infections (Nandagopalan *et al*., 2011). Another species *P. crassipes* has antimicrobial activity against *Klebsiella pneumoniae*, *Proteus* species, *Pseudomonas aeruginosa* and *Staphylococcus aureus* (Mustapha *et al*., 2007; Aliyu *et al*., 2008) etc. Ibekwe *et al*. (2012) have found that *P. crassipes* leaf extracts have antimicrobial activity against *Mycobacterium tuberculosis*. Bello *et al*. (2011) isolated a flavonoid (quercetin-3-O-rutinoside) from *P. crassipes* leaves that was found to be responsible for antimicrobial activity against *E. coli*, *Streptococcus pyogenes*, *Corynebacterium ulcerans*, *Klebsiella pneumoniae*, *Neisseria gonorrhoeae* and *Pseudomonas aeruginosa*. *P indica* has not been reported to possess any anti-microbial activities earlier. Our studies described above suggest that *P. indica* does possess appreciable antimicrobial activities against Gram-positive bacteria.

In disc diffusion tests, the extracts of *P. indica* leaves showed bactericidal activities against the Gram-positive *B. subtilis* bacteria but no detectable activity against Gram-negative bacteria (*E. coli*). The Gram-negative bacteria are generally regarded more resistive due to the presence of lipopolysaccharides in their outer membranes that tend to prevent the entry of inhibitors (Nikaido and Vaara, 1985). MIC results indicate that the antimicrobial components extract similarly in water and methanol; however, MBC results indicate that while these components possess clear bactericidal effects against *B. subtilis* they extract somewhat better in methanol than in water (as evidenced by lower MBC values of methanol extracts).

The heat stability exhibited by these antibacterial activities indicate the plant's leaf tissues as valuable source for extraction of antimicrobial compounds, having application as preservatives in food-processing industries to inhibit the microbial growth in processed food products. Presence of flavonoids in phytochemical testing is interesting as some of the antimicrobials characterized from other plant sources have been found to be flavonoids in nature (Harborne and Baxter, 1999; Alarcón *et al*., 2008; Kanwal *et al*., 2009; Galeotti *et al*., 2008; Sathiamoorthy *et al*., 2007).

Our TLC-bioautography results seem to suggest that antibacterial compounds may be polar in nature as several components in the leaf extracts resolved into areas on TLC plates that exhibited clear bactericidal zones (Fig. 1) when the TLC experiments were carried out in polar solvent system such as ethyl acetate : methanol : water (EMW). In contrast when the same extracts were run in a non-polar solvent (BEA) system on TLC, these bands did not migrate well supporting the view that the antimicrobial components may be more polar in nature. It is not possible to determine at this point whether the different spots (i.e. zones of killing/growth inhibition) found on TLC-bioautography experiments are the parts of the same antimicrobial molecule at different stages of metabolic synthesis or there are multiple compounds of antimicrobial nature in the leaf extracts. Further studies are needed to answer such questions.

CONCLUSION

Based on the results described above, we conclude that the extracts of leaves of this plant *Pavetta indica* possess clear bactericidal activity on Gram-positive bacteria and growth inhibitory activity on Gram-negative bacteria. Since the antimicrobial activities are heat stable as revealed by our heat stress experiment, the compounds responsible for such activities can be considered for use in the food processing industry as preservative.

Further studies will be needed to characterize the antibacterial activities of the components found in the leaf extracts before it is determined whether they can be used in human health care. We have not come across any previous report for the presence of antimicrobial activities in the extract of this species and is the first report of existence of this type of activity in *Pavetta indica*.

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