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Full Length Research Paper

Antimicrobial activity of *Spondias pinnata* resin

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The antimicrobial activity of resin of *Spondias pinnata* Kurz. (Anacardiaceae) was evaluated on *Saccharomyces cerevisiae*, *Bacillus subtilis*, *Escherichia coli*, *Enterobacter sakazakii* and *Acinetobacter baumannii.* Resin extracts were made in different organic solvents. *In vitro* antimicrobial activities of resin extracts were studied by disc diffusion method and macrodilution assays. The microorganism most susceptible to the resin extract was found to be *B. subtilis*, a Gram (+) bacteria. The extracts did not inhibit the growth of Gram (-) bacteria and *S. cerevisiae*, a fungus. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were between 0.5 – 1.0 mg/ml for *B. subtilis* with extracts obtained in petroleum ether. The thermal stability tests showed that the antimicrobial activity found in the extracts was heat stable. In the phytochemical tests, phytosterols and flavonoids gave positive results; carbohydrates and fixed oil also may be present. Saponins, tannins and proteins appeared to be absent. Bioautography experiments revealed the presence, in resin extracts, of one major component and four minor components which inhibited the growth of Gram (+) bacteria.

Key words: Antimicrobial activity, antibacterial activity, MIC, MBC, flavonoids.

INTRODUCTION

Resin is a hydrocarbon secreted by many plants, particularly coniferous trees, valued for its chemical constituents and uses such as in varnishes and adhesives. Plants produce resins for various reasons whose relative importance is debated. It is known that resins seal plant's wounds, kill insects and fungi, and also allow the plant to eliminate excess metabolites. Selection of the plant *S. pinnata* studied in this report was made on the basis of our observation that secretions from its wounds harden slowly into a resinous substance and that the resin is eaten by birds and monkeys. *S. pinnata* is known for its common use in the treatment of infectious diseases like bronchitis, ulcer, diarrhea, dysentery and skin diseases (Melendez and Capriles, 2006; Grosvenor et al., 1995; Valsaraj et al., 1997; Hout et al., 2006). This plant is found in India, Sri Lanka, Burma, China (South, Hainan), Malaysia, Thailand, Laos, etc. The unripe fruits are often used for making pickles. The young leaves, flowers and fruits are edible. Its roots, bark, leaves are useful and its fruits are also used in traditional medicine (Badoni and Bisht, 2009; Gardner et al., 2000).

Earlier studies have shown that the ethanolic extracts of pulp of *S. pinnata* Kurz. have antibacterial activity against *Staphylococcus aureus, E. coli* and *Pseudomonas aeruginosa* (bacteria) and antifungal activity against yeast *Candida albicans* and fungus *Aspergillus flavus* (Keawsa-ard and Liawruangrath, 2009). Bark extract of *S. pinnata* has been reported to possess antioxidant activity (Hazra et al., 2008). A crude extract of *S. pinnata* has been reported to show antibacterial activity (Bibitha et al., 2002). But we have not come across any reports suggesting the presence of any antimicrobial activity in the resins of this plant and felt it worthwhile to investigate the resinous substances for biological activities, particularly in view of our observation that wild animals eat the resins which may be because of

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Table 1. Antimicrobial activities of extracts of *S. pinnata* resin.

Solvent extracts	Zone of inhibition (mm)				
	Microorganisms				
	B. subtilis	***E. sakazakii***	***E .coli***	***A.baumanni***	***S. cerevisiae***
Methanol	7.0	-	-	-	-
Ethanol	7.75	-	-	-	-
Ethyl acetate	8.75	-	-	-	-
Chloroform	8.0	-	-	-	-
Benzene	8.75	-	-	-	-
Petroleum ether	9.0	-	-	-	-
Hexane	8.75	-	-	-	-
Ampicillin (250 µg/ml)	23.5	16.0	S	S	NA
Fluconazole (1 mg/ml)	NA	NA	NA	NA	31.5
DMSO	-	-	-	-	-

- = No activity, S = Slight activity. *B. subtilis* is a Gram (+) bacteria; *E. sakazakii, E. coli* and *A. baumanni* are Gram (-) bacteria. Dried resin extracts obtained from seven different solvents (underlined in left most column with most polar solvent at top to least polar solvent at bottom) were dissolved in DMSO at a concentration of 250 mg/ml and tested by disc diffusion method. Ampicillin and fluconazole were used as positive controls for the bacterial and fungal disc diffusion tests (NA = Not applicable). DMSO, the solubiliser for dried extracts, was used as a negative control.

its remedial properties.

MATERIALS AND METHODS

Collection and identification of plant materials

Fresh resin of *S pinnata* was collected from Santiniketan, West Bengal, India in August, 2009. The plant *Spondias pinnata* (Linn. f.) Kurz. (Syn. *Mangifera pinnata* Linn. f.), which is found abundantly in this locality, was identified by Prof. K 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 organic solvents extracts

Fresh resin material secreted by the plant was air dried under shade and then ground into fine powder with the help of a grinder. For extraction of the dried powder, seven different organic solvents (that is. methanol, ethanol, ethyl acetate, chloroform, benzene, petroleum ether and hexane, in order of decreasing polarity) were used (Table 1). Air-dried powder of resin (5 g) in known volume of solvents (ratio was 1:5 w/v) was taken in a conical flask and kept at room temperature for 10 d with occasional vortexing. 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. It is important to note that, unless mentioned otherwise, the dried extracts obtained separately from organic solvents stated above were again dissolved in DMSO before use in experiments.

Microorganisms for screening

The dried solvent extracts dissolved in DMSO (unless specified \otherwise) were screened against several bacterial strains that is. *E. coli*, *B. Subtilis* (MTCC121), *Enterobacter sakazakii* (MTCC 9868), *Acinetobacter baumannii* (MTCC 9869) and one fungal strain (*S. cerevisiae*) as stated in the respective tables and figures.

Preparation of Inoculums and other microbiological methods

Stock cultures of the microorganisms were maintained at 4°C on slant of LB (Luria-Bertani)-agar and YPD (Yeast extract powder-Mycological peptone-Dextrose)-agar. In general, five bacterial strains were grown in LB-broth while the fungus was grown in YPD-broth. Active and healthy cultures for experiments were prepared by transferring a single colony from the stock culture to 1.5 ml of LB broth (for bacteria) or YPD broth (for fungi) and incubated at 180 rpm for 24 hr at 37 °C (for bacteria) or at 180 rpm for 24 hr at 30°C (for fungus).

The inoculums, used for the experiments, was a broth of each microbial species grown for 24 hr and diluted in 0.9% saline to a final concentration of approximately 10^8 cfu/ml (0.5 McFarland scale). The microbial techniques and compositions of LB or YPD broth and LB or YPD-agar are described in Sambrook and Russel (2001).

Antibacterial and antifungal assays

The disc diffusion method (Bauer et al., 1966), using LB-agar and YPD-agar plates, was employed to screen for antimicrobial activities. The plates were prepared by pouring 30 ml of media into sterile 90 mm Petri dish. Within 15 min 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 incubated at 37°C for 24 hr (for bacteria) or 30°C for 48

h (for fungus). Subsequently, the inhibition zones formed around the discs were measured in millimeter. Ampicillin (at a concentration of 250 µg/ml) and commercial fluconazole, an antifungal (at a concentration of 1 mg/ml), were used as positive controls for all such experiments.

Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

The methods of Greenwood (Greenwood, 1989) were used to determine the MIC and MBC of the extracts. The dried extracts (obtained from petroleum ether) were dissolved in DMSO at a concentration of 250 mg/ml and serial dilutions of it in sterile LB or YPD broth were prepared to obtain extract concentrations of 2, 1, 0.5, 0.25 and 0.125 mg/ml. Then overnight grown test organism (10 µl) was pipetted into each of these test tubes containing 1 ml of the diluted extract (in LB or YPD as described above) and incubated at 30°C for 18 - 20 h. The MIC was recorded as the least concentration of plant extract that completely inhibited the growth of the test organism. The MBC was determined by sub culturing the contents of the tubes for 18 - 20 h to determine the bactericidal activity. The MBC was demonstrated when no growth occurred on the sub cultured medium.

Thermal stability test

To determine the effect of temperature on the stability of extracts, 1.5 ml microfuge tubes, each with 250 mg/ml concentration of the crude extracts in DMSO, were kept 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.

Phytochemical analysis of resin of *S. pinnata*

Qualitative phytochemical tests of *S. pinnata* resin extracts were carried out for detecting the presence of saponins (by foaming test), carbohydrates (by Fehling's test), proteins and free amino acids (by Biuret method), fixed oils (by spot test) etc. Tannins, phytosterols, flavonoids etc. were detected by colour development tests as described by Harborne (1991; 1998) and Khandelwal (2000).

Thin-Layer Chromatography (TLC)

Kieselgel GF254 plates (Merck), 20 X 20 cm^2 and 1 mm thick, were used for TLC. Resin extracts dissolved in petroleum ether at a concentration of 25 mg/ml and were applied on TLC plates. The chromatograms (in duplicate) were run using benzene: ethanol: ammonia (18:2:0.2) as running solvent. At the end of the run, spots and bands on TLC plates were visualized under normal or UV lights after spraying with H_2SO_4 :C_2H_5OH (1:10) reagent.

Bioautography

Bioautography technique of Nostro et al. (2000) was used with some modifications for the detection of antimicrobial components present in the resin extracts, after first separating these components on TLC plates. TLC plates were run as above with resin extracts spotted on it. At the end of TLC run, inoculums of *B. subtilis* containing 10^6 cfu/ml in LB agar were poured on these TLC plates. After solidification of the LB-agar medium on the TLC plates, they were incubated for 24 h at 30°C. Subsequently, the bioautogram was sprayed with a 1% aqueous solution of 3-(4-5-Dimethyl-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and incubated at 30°C for 1 - 4 h. 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

Antimicrobial activities have been reported in the extracts of different parts of *S. pinnata* by other authors (Keawsa-ard and Liawruangrath, 2009; Bibitha et al., 2002). However, to our knowledge no one has described the presence of antimicrobial activities in the resins secreted by this plant.

The resins secreted by the plant have been found to be eaten by monkeys and birds. It is possible that the resins have some healing/curing properties for which they are ingested by wild animals. In order to investigate the antimicrobial activities, if any, of *S. pinnata* resin, its extracts were tested on several different microorganisms stated earlier.

Analysis of antimicrobial activity of resin from *S. pinnata*

The resin extracts of the plant, obtained with seven different organic solvents, showed antimicrobial effects against Gram (+) bacteria such as *B. subtilis* (Table 1). Extracts from the resin inhibited the growth of another Gram (+) bacteria *Microbacterium testaceum* (MTCC 9870; data not shown, Gupta and Roy, 2010;Unpublished observation) also. It was also observed (Table 1) that among these solvents used for extraction, resin extract obtained with petroleum ether showed best activity against *B. subtilis,* that is, it showed an inhibition zone of 9 mm (diameter) at a crude extract concentration of 250 mg/ml, in disc diffusion test. The resin extracts were also tested on three Gram (-) bacteria; *E. coli, E. sakazakii* and *A. baumannii* and one fungus (*S. cerevisiae*) by the disc diffusion method (Table 1) and found to have no detectable activity against them. The reason for the difference in sensitivity between Gram (+) and Gram (-) bacteria could be explained by the morphological difference between these microorganisms. Gram (-) bacteria have an outer phospholipid membrane carrying the structural lipopolysaccharide components. This makes the cell wall impermeable to lipophillic solutes such as some plant products (Nikaido and Vaara, 1985). The Gram (+) bacteria having only an outer peptide-glycan layer, which is not a selective permeable barrier (Scherrer and Gerhardt, 1971), should be more suscep tible to drugs. For the sake of simplifying the experimental

Table 2. MIC and MBC values of resin extract of *S. pinnata* against *B. subtilis*.

Bacteria	MIC (mg/ml)	MBC (mg/ml)
B. subtilis	0.5 - 1	0.5 - 1

The resin extract used in this experiment was obtained from petroleum ether extraction. The dried extract was dissolved in DMSO (250 mg/ml) for the MIC and MBC tests.

Table 3. Thermal stability test of resin extract of *S. pinnata* against *B. subtilis*.

Thermal treatment	Zone of inhibition (mm)
Normal	8
40°C	8
80°C	8
100°C	7
Autoclave	7.5
Ampicillin (Autoclave)	21.5
DMSO	-

- = No growth, DMSO and Ampicillin (250 µg/ml) have been used as –ve and +ve controls respectively. The resin extract in this experiment was obtained from petroleum ether extraction. The dried extract was dissolved in DMSO for the thermal stability test.

hazards of working with pathogenic microbes, all subsequent studies with resin extracts were done with *B. subtilis* only.

MIC and MBC tests

MIC and MBC results are shown in Table 2 from which it may be concluded that the concentrations between 0.5 - 1 mg/ml of crude resin extract of the plant have the equivalent MIC and MBC values. MBC results indicate that the resin of *S. pinnata* extract have bactericidal effects.

Thermal stability test

Results showed that there were slight losses of anti-microbial activity of the resin extracts at higher temperature. For the positive control ampicillin, which was dissolved in DMSO and autoclaved like the experimental samples, very little loss in activity was detected. So, it may be concluded that the structural conformation of the compounds from the resin extract, which was dissolved in DMSO, was only slightly affected by the heat at 100°C or above that is, in autoclave, and they appear to be heat stable (Table 3).

Table 4. Preliminary qualitative phytochemical analysis of resin extract of *S. pinnata*.

Phytochemical	Results
Saponins	-
Phytosterols	+
Flavonoids	+
Tannins	-
Carbohydrates	-/+
Protein and free amino acids	-
Fixed oil and fats	-/+

+ = Present; - = Absent; -/+ = May be present. The resin extract in this experiment was obtained from petroleum ether extraction. The dried extract was used directly for phytochemical analysis.

Phytochemical analysis

Results of qualitative phytochemical analysis (Table 4) showed the presence of phytosterols and flavonoids in the petroleum ether extract of resin of S. *pinnata.* But from these phytochemical analyses it was not possible to determine which of the phytochemical/s is/are responsible for the antimicrobial activity detected in the resins. The effect may be due to a single compound or may be a combined effect of different compounds present in the extract of that sample. It is to be noted, however, that flavonoids have indeed been found to be responsible for antimicrobial activities in other systems (Chaturvedi et al., 2010; Rattanachaikunsopon and Phumkhachorn, 2010).

TLC and bioautography tests

The results of thin layer chromatography (TLC) and bioautography experiments carried out with a moderately non-polar solvent system using benzene: ethanol: ammonia (18:2:0.2) are shown in Figure 1. Lanes A, B and C indicate that separation of various components in the resin extract has taken place. Growth inhibition zones, indicated in lane D, show the presence of active bactericidal components, well separated from each other, in at least one major and four minor positions of bioautogram.

DISCUSSION

Selection of the plant *Spondias pinnata* studied in this report was made on the basis of our observation that secretions from its wounds hardens slowly into a resinous substance and that the resin is eaten by birds and monkeys. Based on the results described above, we conclude that the extract of resin of this plant possesses discrete antimicrobial activity on Gram (+) bacteria. It is to

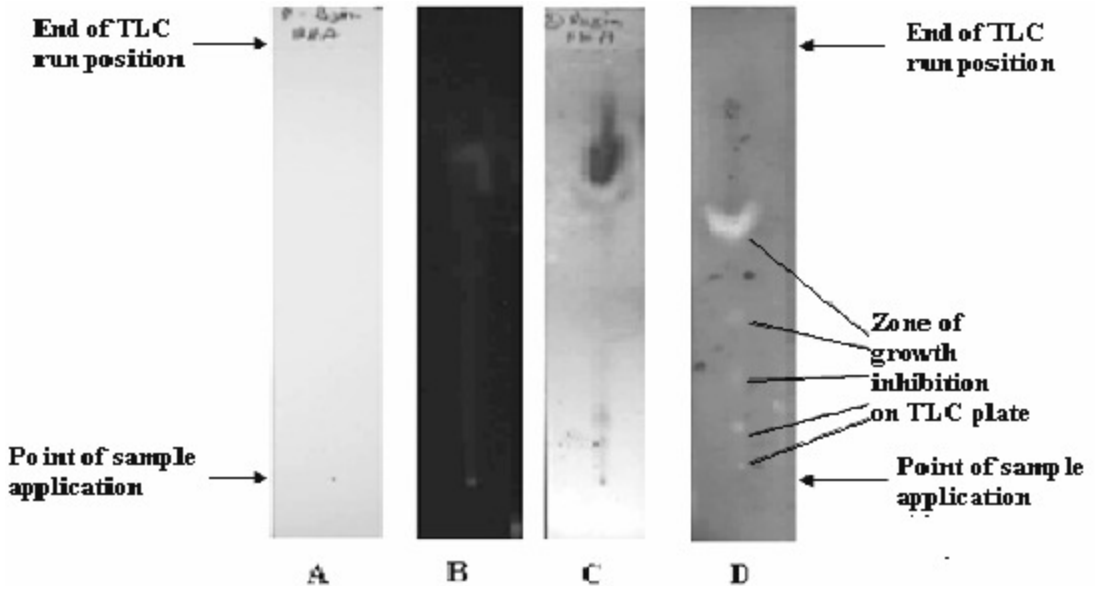

Figure 1. TLC (Lanes A, B and C) and bioautograph (Lane D) of resin extract of *S. pinnata.* Resin extracts made from petroleum ether (at a concentration of 25 mg/ml) were spotted (5 µl) on TLC plates. Running solvent system used for TLC was benzene: ethanol: ammonia (18:2:0.2). (A) In normal light, no band is visible. (B) In long wave UV, two bands are visible, one on starting point and another towards top position. (C) TLC plate after spraying with H_2SO_4 and Ethanol solution showing multiple spots/bands. (D) After staining bioautograph with MTT, one faster moving major bactericidal band and four slower moving minor bactericidal spots appear. The lines drawn in D indicate these zones of growth inhibition, created because of bactericidal components present in *S. pinnata* resin.

be noted that some previous researchers (Keawsa-ard and Liawruangrath, 2009) reported that the pulp extract of this plant has anti-Gram (-) bactericidal activity. However, in our studies this activity was not found. It may be possible that the resin of this plant contains different kinds of antimicrobial constituents than its pulp extract. Only further studies can clear up this issue. Results of bioautography experiments showed the presence of multiple bactericidal components in the resin extract of this plant with one major and four minor growth inhibition areas on the bioautograph. However, it is not presently possible for us to indicate whether the minor components are smaller units of the same major component or they are different from each other. Phytochemical analyses reveal the presence of falvonoids and phytosterols in the resin extracts. Although flavonoids have been found to be responsible for the observed antimicrobial activity in other systems (Chaturvedi et al., 2010; Rattanachaikunsopon and Phumkhachorn, 2010), more studies will be needed to determine the nature of active compounds found in the resin of *S. pinnata.* We will conduct further research to isolate the active compound/s responsible for the antimicrobial activities that we have observed in the resin extract of this plant. Along with this, the plant resin can be tested for different other disease causing microorganism in higher animals like mice and if it is not having any adverse effect on the animal body, then it can be taken as medicine after clinical trials.

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REFERENCES

Bauer AW, Kirby WMM, Sherries T (1966). Antibiotic susceptibility testing by a standardsingle disc method. Am. J. Clin. Pathol., 45: 493-496.

Bibitha B, Jisha VK, Salitha CV, Mohan S, Valsa AK (2002). Antibacterial activity of different plant extracts. Ind. J. Microbiol., 42: 361-363.

Badoni A, Bisht C (2009): Importance and Problems in Natural Regeneration of *Spondias pinnata*. Report Opinion, 1(5): 12-13.

Chaturvedi A, Singh S, Nag TN (2010). Antimicrobial activity of flavonoids from *in vitro* tissue culture and seeds of Gossypium species. Rom. Biotechnol. Lett. 15: 201

Gardner S, Sidisunthorn P, Anusarnsunthorn V (2000): A field guide to Forest Trees of Northern Thailand. Kobfai Publishing Project, Bangkok, Thailand.

Greenwood D (1989). Antibiotic sensitivity test In: antimicrobial chemotherapy, (Oxford University press, New York) 91.

Grosvenor PW, Supriono A, Gray D (1995). Medicinal plants from Riau province, Sumatra, Indonesia. Part 2: Antibacterial and antifungal

activity. J. Ethnopharmacol., 45: 97-111.
Harborne J B (1998). Phytochemical methods: a guide to modern techniques of plant analysis. Chapman and Hall, London, 302.
Harborne J B (1991). Phytochemistry methods. London, Chapman and Hall, p. 228.
Hazra B, Biswas S, Mandal N (2008). Antioxidant and free radical scavenging activity of *Spondias pinnata*. BMC Complement. Altern. Med. 8: 63-72.
Hout S, Chea A, Bunn SS, Elias R, Gasquet M, Timon-David P, Balansard G, Azas N (2006). Screening of selected indigenous plants of Combodia for antiplasmodial activity. J. Ethnopharmacol. 107: 12-18.
Keawsa-ard S, Liawruangrath B (2009). Antimicrobial Activity of *Spondias pinnata* Kurz. PACCON (Pure and Applied Chemistry International Conference).
Khandelwal KR (2000). Practical Pharmacognosy.Techniques and Experiments, Nirali Prakashan, Pune, 2: 149.
Melendez PA, Capriles VA (2006). Antibacterial properties of tropical plants from Puerto Rico. Phytomedicine 13: 272-276.
Nikaido H, Vaara M (1985). Molecular basis of bacterial outer membrane permeability. Microbiol. Rev., 49: 1-32.
Nostro A, Germano MP, Angelo VD, Marino A, Cannatelli MA (2000). Extraction methods and bioautography for evaluation of medicinal plant antimicrobial activity. Ltt. Appl. Microbiol., 30: 379-384.
Rattanachaikunsopon P, Phumkhachorn P (2010). Contents and antibacterial activity of flavonoids extracted from leaves of *Psidium guajava*. J. Med. Plant. Res. 4: 393-396.
Sambrook J, Russel DW (2001). Molecular cloning, a laboratory manual. 3rd edition. Cold spring harbor laboratory press.
Scherrer R, Gerhardt P (1971). Molecular sieving by the *Bacillus megaterium* cell wall and Protoplast. J. Bacteriol., 107: 718-735.
Valsaraj R, Pushpangadan P, Smitt UW, Adsersen A, Nyman U (1997). Antimicrobial screening of selected medicinal plants from India. J. Ethnopharmacol. 58: 75-83.