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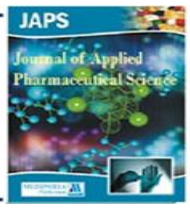

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Leaf and Seed extracts of *Bixa orellana* L. exert anti-microbial activity against bacterial pathogens

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ABSTRACT

Methanol extracts of the leaf & seed of *Bixa orellana* L. were studied for *in vitro* antimicrobial activity against MTCC strains of *Staphylococcus aureus, Salmonella typhi, Klebsiella pneumoniae, Pseudomonas aeruginosa, Enterococcus fecalis, Vibrio cholera, Moraxella catarrhalis, Acinetobacter* sp., *Brucella* sp. along with fungal pathogens *Candida albicans, Aspergillus niger* and the dermatophytes *Trichophyton mentagrophytes* & *Trichophyton rubrum*. Leaf extract of *B. orellana* at 1000 µg/ml concentration showed significant inhibition against all the tested bacteria and fungus, with highest inhibition zone (18±0.3 mm) against *S. typhi, Acinetobacter* sp., *T. mentagrophytes* and *T. rubrum*. Seed extract of *B. orellana* was comparitively less efficacious in most of the tested pathogens, except *Brucella* sp. which was appreciably inhibited (15±0.1 mm). Minimum Inhibitory Concentration (MIC) of leaf extract was determined as 15.62 µg/ml against *S. aureus* and 31.25 µg/ml for *K. pneumoniae, P. aeruginosa, E. fecalis* & *S. typhi*, on average. Among the dermatophytes, 78.2% inhibition was seen in *T. mentagrophytes* & *T. rubrum*. Scanning Electron Microscopic (SEM) studies of the treated *P. aeruginosa* cells revealed disintegration & aggregation of cells after treatment with the leaf extract. Phytochemical analysis of leaf & seed extracts suggested the presence of flavonoids, tannins, saponins, steroids. Alkaloids were detected only in the leaf & anthroquinones, in the seeds.

Keywords: *Bixa orellana* L., antibacterial, antifungal, SEM & phytochemical analysis

INTRODUCTION

In developing countries, people of native communities use *Bixa orellana* L. commonly known as 'achiote/ annatto'(Family: Bixaceae)'(Gamble, 1957) as folk medicine in the form of decoctions, teas & juices for the treatment of common infections. In Philippines, the leaf decoction is used to cure skin diseases and burns. The leaves are a popular febrifuge in Cambodia. The infusion of leaves is prescribed as a purgative and in the treatment of dysentery. In Central America, the oil derived from seeds is used to cure leprosy and decoction is given to treat jaundice[4]. The species is used medicinally in various parts of the world & cultivated in warmer regions like India, Sri Lanka and Java, exclusively for the dye obtained from the seeds. In India, the plant is cultivated especially in western parts of the country. A colored compound obtained from the pulp of the seeds called 'bixin' is used all over the world as a red-orange dye, for coloring rice, cheese, soft drinks, oil, butter and soup. The dye is also used in some regions in textile industry and the seeds, as a condiment (Parekh *et al.*, 2005; Metta Ongsakul *et al.*, 2009). Ayurveda practitioners in India use it as an astringent and mild purgative and are considered as a good remedy for treating dysentery and kidney diseases. The root bark is anti-parasitic and antipyretic. The traditional healers claim that *Bixa* sps are more efficient to treat infectious diseases than synthetic antibiotics.

Even though hundreds of plant species have been tested for antimicrobial properties, the vast majority of them have not yet been critically evaluated (Erdogrul, 2002). Preliminary pharmacological screening of antibacterial activity of *B. orellana* leaves and seeds has been done (Jamil Ahmad Shilpi *et al*., 2006; Fleisher *et al*., 2003). Our work demonstrates a thorough elucidation of the antimicrobial activity of *B. orellana* & subsequent electron microscopy to partially understand the mode of action of the crude extracts.

MATERIAL AND METHODS

Plant Collection & Extraction

The fresh leaves and seeds of *B. orellana* L. were collected from Agricultural University, Coimbatore, Tamil Nadu, India (30th January 2011). They were shade dried and pulverized. 25 g of the powder was subjected to soxhlet extraction with 250 ml of methanol and concentrated to dryness in vacuum (Buchi Rotavapor R-200®). The dried extract was dissolved in 0.25% Dimethyl Sulphoxide (DMSO, Merck®) to a concentration of 100 mg/ ml & the working concentration, from 1mg/ ml.

Test Organisms

The microorganisms used for the biological evaluation were purchased from the Microbial Type Culture Collection & Gene Bank (MTCC), Chandigarh. Among the Gram+ve bacteria *Staphylococcus aureus* MTCC 3160 and *Enteorcoccus fecalis* MTCC 3159 and Gram-ve bacteria *Klebsiella pneumonia* MTCC 3384, *Pseudomonas aeroginosa* MTCC 2582,*Vibirio cholera* MTCC 3906, *Moraxella catarrhalis* MTCC 445, *Acinetobacter sp.* MTCC 1611, *Aeromonas hydrophila* MTCC 1739, *Brucella sp.* MTCC 685 and *Salmonella typhi* MTCC 3216 were tested. Among the fungal pathogens, *Aspergillus niger* MTCC 6484, *Trichophyton mentagrophytes* MTCC 7250, *Trichophyton rubrum* MTCC 3272 and *Candida albicans* MTCC 1637 were tested. Fungal strains were grown in Potato dextrose agar (Merck®) and Sabouraud's glucose agar (Merck®) and maintained by periodic sub culturing process.

Preparation of Inoculum

Stock cultures were maintained at 4°C on nutrient agar slants. Active cultures for experiments were prepared by transferring a loopful of cells from the stock cultures to Mueller-Hinton broth (MHB) for bacteria and Sabouraud Dextrose Broth (SDB) for fungi and were incubated for 24 h at 37°C and 25°C respectively. To 5ml of MHB and SDB 0.2 ml of respective cultures were inoculated and incubated till it reached the turbidity equal to that of the standard 0.5 McFarland solution at 600 nm which is equivalent to 10^6-10^8 CFU/ml (Irobi *et al.*, 1996).

Preparation of extracts

Stock solutions of the extracts and controls were prepared in dimethyl sulphoxide (DMSO, Merck®) at a concentration of 100 mg/ml, respectively, resulting in homogeneous solutions. Further dilutions were performed using sterile distilled water. Positive controls were prepared with streptomycin (10 µg/disc, Sigma®).

Preliminary Phytochemical Screening

Phytochemical screening of the plant extracts were carried out as per the methods given by Kokate C.K 1986, to observe the presence of various phytochemicals (Mohammed *et al*.,2010)

Antimicrobial Assay

Disc Diffusion (Kirby-Bauer) method was followed for antibacterial assay. *In vitro* antimicrobial activity was screened using MHA. The MHA plates were prepared by pouring 18ml of molten media into sterile petriplates. The plates were allowed to solidify for 5 min and 0.1 % inoculum suspension of tested organisms was swabbed uniformly and the inoculum was allowed to dry for 5 min. Different concentrations of the extracts (1000, 500, 250, 125µg/ disc) were loaded on 5 mm sterile individual discs (HiMedia®) & thoroughly dried in air draft to remove traces of the solvent. Negative control was prepared using respective solvent. Streptomycin (10 µg/disc) was used as positive control. The fortified discs were placed on the surface of medium using a disc template and incubated at 37°C for 24 hr. Inhibition zones formed around the discs were measured with transparent ruler (in millimeters)(Kumaraswamy et al ., 2002).

Antifungal assay was performed by the standard procedure (Hari Babu *et al.*, 2011). 1 ml of methanol extracts of leaves and seed of *B. orellana* was added to 100 ml of Potato Dextrose Agar (Himedia®) medium and poured into sterile petriplates. After solidification, a loop full of culture was placed on the centre of the plate. Controls were maintained with DMSO. The plates were incubated at 29ºC. Growth was monitored for 24, 48 and 72 hr, depending on the period of incubation time required for the visible growth i.e. 24 hr for *C. albicans*, 48 hr for *A. niger* and 72 hr for the dermatophytes. The growths of treated samples were compared with their respective control plates.

Minimum Inhibitory Concentration (MIC)

Selected plant extracts were subjected to a serial dilution (1000 µg-1.906 µg) in sterile nutrient broth medium. 96-well titre plate was used for the assay, in which, 20 µl of the culture organism and 20 µl of selected plant extract were loaded and incubated at 37ºC for 24 hr. The highest dilution of the plant extract that retained its inhibitory effect resulting in no growth (absence of turbidity) of a microorganism is recorded as the MIC value of the extract. DMSO control was maintained in parallel.

Scanning Electron Microscopy (SEM)

Methanol leaves extract was added to serially diluted (10^{-4}) cells of *P. aeruginosa* (one of the susceptible organisms) & incubated at 37ºC/24 h. After incubation, the cells were centrifuged at 6000 rpm for 15 min and washed twice with 0.01 M potassium phosphate buffer (pH 7.0). The samples obtained in the form of pellets after centrifugation at 6000 rpm were fixed with 2% glutaraldehyde for 2 hr at 4° C. The pellets thus obtained were

dehydrated in a gradient ethanol (10-100%). Up to 40% ethanol centrifugation carried out after which the cells were transferred onto the slide and treated up to 100% ethanol followed by drying the slides in desiccators. The slides were then subjected to scanning electron microscopy on a scanning electron microscope (LEOL® 6360, Japan, Coater: JEOL-FC® 1600 Auto coater) (McDougall *et al.*, 1994).

RESULTS

Preliminary Phytochemical Analysis

Preliminary phytochemical analysis carried out on the crude methanol extract indicated the presence of steroids, flavonoids, tannins, saponins and proteins in both leaf & seed extracts of *B. orellana.* (Table 1).

Table 1. Phytochemical analysis of leaf and seed extracts of *B. orellana* L.

S.No	Phytochemicals	Leaves	Seeds
1.	Tannins	+	+
2.	Saponin	+	+
3.	Flavonoid	+	+
4.	Alkaloids	+	-
5.	Proteins	+	+
6.	Steroids	+	+
7.	Anthroquinone	-	+

Note: + : Presence, - : Absence.

Table 2. Antimicrobial activity of leaves and seed extracts of *B. orellana* L.

S. No.	Organisms	Leaf Extract				Seed extract				Control
		1000 µg/ml	500 µg/ml	250 µg/ml	125 µg/ml	1000 µg/ml	500 µg/ml	250 µg/ml	125 µg/ml	10µg/disc
1.	*S. aureus*	16±0.1	13±0.1	9±0.2	8±0.1	13±0.2	12±0.5	10±0.5	6±0.2	20±0.1
2.	*E. faecalis*	14±0.1	13±0.2	11±0.2	10±0.2	14±0.2	12±0.3	10±0.4	7±0.1	17±0.2
3.	*V. cholerae*	15±0.3	13±0.2	12±0.1	9±0.2	7±0.3	6±0.3	7±0.2	6±0.2	20±0.2
4.	*K. pneumoniae*	16±0.2	14±0.1	13±0.1	10±0.2	7±0.3	6±0.1	5±0.2	-	24±0.1
5.	*P. aeruginosa*	16±0.1	14±0.2	12±0.3	10±0.3	11±0.1	10±0.1	10±0.2	6±0.2	25±0.3
6.	*M. catarrhalis*	13±0.3	11±0.3	10±0.3	8±0.2	10±0.2	7±0.2	6±0.1	-	25±0.2
7.	*Brucella* sp.	12±0.2	13±0.1	10±0.2	10±0.5	15±0.1	14±0.1	12±0.1	6±0.5	16±0.5
8.	*Acinetobacter* sp.	18±0.2	15±0.3	10±0.1	9±0.1	10±0.1	9±0.3	7±0.3	7±0.5	20±0.2
9.	*A. hydrophila*	12±0.3	10±0.5	8±0.2	7±0.3	7±0.5	-	-	-	23±0.2
10	*S. typhi*	18±0.3	14±0.3	13±0.2	9±0.2	7±0.3	6±0.5	6±0.3	5±0.2	9±0.2

Note: Control: Streptomycin 10µg/ disc. Triplicates were maintained for all the organisms. The average of triplicates was tabulated.

Antimicrobial Assays

Both leaf & seed extracts of *B. orellana* exhibited dose-dependent antimicrobial activity against the tested pathogens. *S. typhi and Acinetobacter* sp. was found to be more sensitive to extracts of leaf (18±0.2 mm) followed by *S. aureus, K. pneumonia* & *P. aeruginosa* (18±0.1 mm) compared to Streptomycin control (21.8±0.3 mm on average). However, the leaf extract showed effective inhibition of *S. typhi*, compared to the control (Table 2.). The blind control (DMSO) exhibited 'nil' inhibition. Methanolic extract of leaf was found to be the most active against the tested fungi, compared with the seed extracts. *T. mentagrophytes* (73.6%) *T. rubrum* (78.1%) were the most sensitive organisms to the leaf extracts followed by *A. niger* and *C. albicans.* Seed extracts showing least inhibitory effects on dermatophytes (38.4 & 28.5%) followed by *C. albicans* (18.1%). The results were tabulated in Table 4.

Minimum Inhibitory Concentration (MIC)

It was significant to find that the MIC values were 15.62 µg/ml & 62.5 µg/ml for leaf & seed extracts respectively, against *S. aureus.* Most of the organisms were inhibited at MIC of 31.25 µg/ml of leaf extract. *A. hydrophila* (MIC: 1000 µg/ml) and *M. catarrhalis* (MIC: 250 µg/ml) were sensitive only at higher concentrations of seed & leaf extracts (Table 3.).

Table 3. MIC of Leaf and Seed extracts of *B. orellana* L.

S. No	Organism	Minimum Inhibitory Concentration (MIC) (µg/ml)	
		Leaf extract	Seed extract
1.	*S. aureus*	15.62	62.5
2.	*E. faecalis*	31.25	62.5
3.	*Brucella* sp.	65.50	125
4.	*V. cholerae*	31.25	250
5.	*K. pneumoniae*	31.25	250
6.	*P. aeruginosa*	31.25	62.5
7.	*M. catarrhalis*	250	250
8.	*Acinetobacter* sp.	31.25	250
9.	*A. hydrophila*	62.5	1000
10.	*S. typhi*	31.25	125

Note: Triplicates were maintained for all the organisms. The average of triplicates was tabulated.

Table 4: *In vitro* antifungal activity of methanol leaf and seed extracts of *B. orellana* L.

S.No	Organisms	Control	Leaf Extract	% inhibition	Seed Extract	% inhibition	Control
1.	*C. albicans*	11±0.5	7±0.2	36.36	9±0.1	18.18	6±0.2
2.	*A. niger*	36±0.2	19±0.1	47.22	35±0.2	2.77	7±0.1
3.	*T. rubrum*	32±0.2	6±0.1	78.12	20±0.2	28.57	10±0.1
4.	*T. mentagrophytes*	19±0.3	5±0.3	73.68	8±0.1	38.46	6±0.2

Note: Control: Amphotericin-B 10µl/ sample. Triplicates were maintained for all the organisms. The average of triplicates was tabulated.

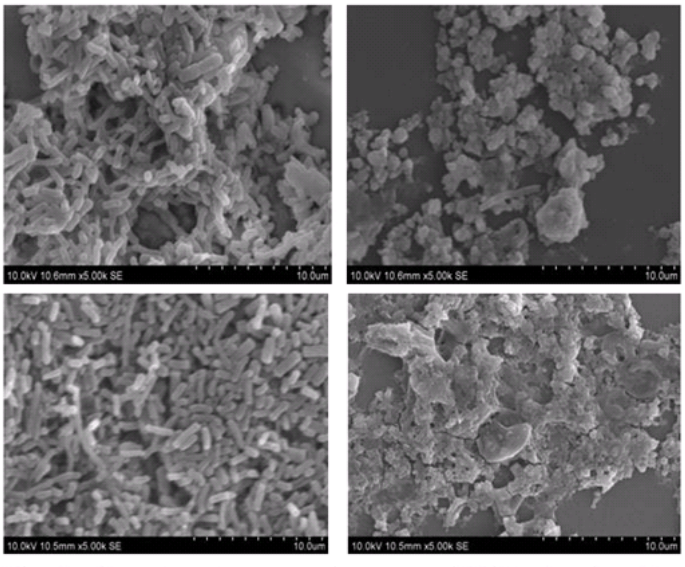

Fig. 1. Scanning electron microscopy (SEM) showing the antagonistic activity of the leaves extract of *Bixa orellana* (A) *P. aeruginosa* untreated, (B) *P. aeruginosa* treated with leaf extract of *B. orellana* (C) *Bacillus subtilus* untreated and (D) *Bacillus subtilus* treated with leaf extract of *B. orellana*

SEM analysis

SEM analysis of *P. aeruginosa* cells treated with 1000 µg/ ml of the leaf extract of *B. orellana* suggested lysis & aggregation, clearly indicating the cell death (Fig.1).

DISCUSSION

In the present study, methanolic leaf extract of *B. orellana*, on average, is 50% more efficacious than the seed extract. This might be due to the absence of alkaloids in the seeds. In another study, crude alkaloid extract of *Mahonia manipurensis* exhibited effective inhibition against *B. cereus, E. faecalis, E. cloacae* & *S. flexneri* (Pfoze *et al.*, 2011). The inhibitory activity of seed extract of *B. orellana* could be attributed to the presence of flavonoids. Flavonoids have the ability to complex with extra cellular & soluble proteins and with bacterial cell walls. Lipophilic flavonoids may also disrupt bacterial membranes (Clements *et al.*, 2002). Flavonoid-rich extracts of seeds and callus tissues of three species of *Gossypium* revealed inhibitory activity against *B. cerus, P. aeruginosa, S. typhimurium* & *S. aureus*, but resistant to *C. albicans*, with maximum activity in the flavonoid fraction of callus tissue as compared to seeds (Chaturvedi *et al.*, 2010). The disintegration & aggregation of *P. aeruginosa* cells treated with *B. orellana* observed by SEM suggests a similar mode of action.(Latha *et al.* 2010) proved that extract of *Vernonia cinerea* (L.) caused severe alterations of the cell wall with the formation of holes, invaginations and morphological disorganization in *P. aeruginosa* cells with an MIC of 3.13 mg/ml. *B. orellana* is also found to contain saponins & steroids. The mode of action of saponins against bacteria is due to its ability to cause leakage of proteins & certain enzymes from the cell (Vila, 2002). Whereas, the sensitivity of steroids & the membrane lipids indicates their specific association that causes leakage from liposomes (Marjorie, 1999). Saponins Gymnemagenol & Dayscyphin-C isolated from *Gymnema sylvestre*, at 50 µg/ml, showed a good cytotoxic activity (63% & 52%) in HeLa cells, at 48 hours (Khanna & Kannabiran, 2009). Further, purification & characterization of the saponins from *B. orellana* is much needed to correllate the structure & activity.

Gram –ve bacteria which are responsible for a large number of infectious diseases have a unique outer membrane that contains lipopolysaccharides which render them impermeable to certain antibacterial compounds (Sathish Kumar Jayaraman *et al.*, 2008). *B. orellana* exerts notable activity against serious pathogens including *S. typhi, V. cholerae, P. aeruginosa* & *K. pneumoniae* etc. Joshi *et al.* (2011) has proved that aqueous ethanolic extract of *Eugenia caryophyllata* was most effective against *S. typhi* (22 mm) and *S. paratyphi* (21 mm). Ethanol extracts of *Terminalia chebula* and *Ocimum sanctum* exhibited antibacterial activity (16 mm) against *K. pneumoniae* (Sharma *et al.*, 2009). Our results on *B. orellana* were comparable to these findings. Extract (0.17-3.0 mg/ml) from *Croton urucurana* Baill. (Euphorbiaceae) inhibited (Zone range: 7.6-26.9 mm) *Tricophyton tonsurans, T. mentagrophytes, T. rubrum, M. canis* & *E. floccossum*, with MIC of 1.25-2.5 mg/ml. In our study, *B. orellana* curbed 78% of the growth of *T. mentagrophytes* and *T. rubrum*. Senthil Kumar & Vinoth Kumar (2011) showed that nitrogenous compound 8-Azabicyclo [3.2.1] octan-3-ol,8-methyl-,endo- isolated from the leaf & root samples of *Withania somnifera* curbed the growth of bacterial pathogens, including *T. rubrum*. It is interesting to note that in our study, leaf extract (alkaloid +ve) of *B. orellana* has profound activity (78%) compared to seed extracts (alkaloid –ve).

Development of resistance to chemotherapeutic agents shown by microorganisms appears to be a continuous process from time immemorial. The result of the present work partially validates the use of *B. orellana* in folk medicine for the treatment of various diseases. It also underlines the ethnobotanical approach for the selection of plants in the discovery of new bioactive compounds. Thus, the study ascertains the value of plants used in ayurveda, which could be of considerable interest to the development of new drugs.

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