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In vitro antibacterial potential of *Bixa orellana* L. against some pathogenic bacteria and comparative investigation on some standard antibiotics

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Abstract

Medicinal plant *Bixa orellana* L. has been traditionally used in several regions of the world to treat a number of ailments including internal inflammation, gastric ulcer and stomach discomfort. The aim of this study was to determine the *in vitro* antibacterial activity of *Bixa orellana* L. against some pathogenic bacterial strains and compare with some standard antibiotics. Methanolic leaf extracts of *Bixa orellana* L. were analyzed for their antibacterial activity against pathogenic bacteria including Gram positive bacteria (*Bacillus subtilis*, *Sarcinalutea*) and Gram negative bacteria (*Escherichia coli*, *Salmonella typhi*, *Shigella dysenteriae*, *Klebsiella pneumonia*, *Proteus vulgaris*) by disc diffusion assay. The zone of inhibition produced by methanolic extracts of *Bixa orellana* L. comparing with standard antibiotics discs showed moderately significant antibacterial activity against almost all of the tested bacterial strains except *Salmonella typhi* which gave promising clear zone (14mm). The methanolic extract showed significantly remarkable results at minimum inhibitory concentrations (MICs) of 5 mg/ml against two Gram positive bacteria, *B. subtilis*, *S. lutea* and one Gram negative bacteria *S. dysenteriae* while four other Gram negative bacteria e.g., *E. coli*, *S. typhi*, *K. pneumonia* and *P. vulgaris* with their MIC value of 10 mg/mL. The study suggests that *Bixa orellana* L. could be a potential antibacterial agent in future.

Keywords: Extract, Methanol, Bacteria, Antibiotics, Leaf.

Introduction

Bixa orellana L. commonly known as annatto belonging to the family Bixaceae is a shrubby tree which ranges from 310 meters in height. Its glossy cordate acuminate leaves are ever green with reddish veins with a thin long petiole. *Bixa orellana* L. is valued for its food and medicinal uses. Different parts of the plant proved to be therapeutic in several ways. The seeds and root barks were used to treat gonorrhoea, the leaves and roots for epilepsy, dysentery, fever, and jaundice [1]. Recently, *B. orellana* has been associated to becoming a good source of antibacterial components against several human pathogens including *Bacillus cereus* [2], *Bacillus pumilus* [3], *Klebsiella pneumoniae* and *Salmonella typhi* [4], *Helicobacter pylori* [5], *Staphylococcus aureus* and *Escherichia coli* [6,7]. The plant has been proved to be an effective antibacterial agent.

Antibiotics, one of the available and important combatants are used in the treatment of infectious diseases [8]. Different antibiotics show their inhibitory efficacy on different pathogenic organisms but availability of a large number of multidrug resistant bacteria has threatened the doctors and researchers now a days.

These natural products may offer a new source for developing new antibacterial agents [9]. Regarding this issue over the last few years, number of studies has been conducted to identify plant derived substances for the treatment of various diseases [10,11]. At present nearly 30% modern medicines are directly or indirectly derived from plants [12]. The study aims to assess the antibacterial activity of *Bixa orellana* L., measure the minimum inhibitory concentration (MIC) of the selected plant extracts against some pathogenic bacteria and to compare the result with commercially available antibiotic drugs.

Materials and Methods

Plant leaf extracts preparation

Leaves of *Bixa orellana* plant was collected during the month of February 2013 from the campus of Jessore University of Science and technology, Jessore, Bangladesh. It was identified by analyzing the characteristics and authenticated from Department of Botany, University of Rajshahi, Rajshahi, Bangladesh.

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The collected plant leaves were washed with water and cut into small pieces and then air dried under shade at room temperature. After that the air dried leaves were pulverized into powder by commercial blender (Philips, South Korea) 150gm of the leaf powder were taken by measuring weight with electric balance in a 500 ml conical flask added with 350ml of the solvent (methanol). Then conical flask was kept on shaker incubator at room temperature for 9 days. Then the plant extract was filtered into beaker through Whatman no.1 to exclude the insoluble leaf powder. The weight of empty beaker was measured before filtering. After filtration, beaker containing plant extract with solvent were kept at room temperature to evaporate solvent. After complete evaporation of solvent, only plant crude extracts were obtained. The crude extracts of methanol solvent (1 gm) were obtained.

Test Organism preparation

Bacillus subtilis IFO 3026, *Sarcinalutea* IFO 3232, *Escherichia coli* IFO 3007, *Proteus vulgaris* MTTC 321, *Klebsiella pneumonia* ATTC 10031 were used in this study, obtained from the Microbiology laboratory of Department of Biotechnology and Genetic Engineering, Islamic University, Kushtia, Bangladesh. Another two microorganisms *Salmonella typhi* and *Shigella dysenteriae* were collected from the laboratory of Department of Microbiology, Jessore University of Science and Technology, Jessore, Bangladesh. Nutrient agar media and Nutrient broth were used for the culture of bacteria in this study. For the antibacterial assay, determination of minimum inhibitory concentration and the preparation of further stock culture, 100 μ L liquid culture was taken from stock culture and inoculated into 125 ml conical flask containing 25 ml Nutrient broth media at 37 °C with continuous shaking at 250 rpm for culturing the bacteria to mid-log phase of absorbance at 600 nm reached at 0.4 by using UV spectrophotometer (T80 UV/VIS spectrophotometer, Oasis scientific Inc., USA) for bacterial broth culture. For antibacterial assay, 100 μ L of starter bacterial culture was spreaded on each Nutrient agar media-plate for the bacterial growth.

Preparation of working disk

The Whatman No. 1 filter paper of 6 mm in diameter was used as discs. These discs were transferred into a small vial and autoclaved at 15 lb/inch² pressure for 15 minutes at 121 °C followed by complete oven-dry at 60 °C. Two hundred (200) mg of crude methanolic extract of *Bixa orellana* L. leaf was dissolved into 10 mL of methanol. Thus the concentration of methanol extract was obtained as 20 mg/ml. Each piece of Whatman no. 1 sterile filter paper disc was impregnated with 10 μ L of 20 mg/ml (200 μ g/disc) of *Bixa orellana* L. leaf extract of methanol. The discs were completely air dried in the laminar flow cabinet and used for antibacterial assay. Blank discs impregnated with methanol solvent were used as negative control.

Determination of Antibacterial Assay

In vitro antibacterial assay was performed by using disc diffusion method. Prepared working disk were placed on nutrient-agar-medium plate which was spreaded with 100 μ L of starter bacterial culture followed by incubation overnight at 37 °C. The culture plates were examined and the zones of inhibition were measured in millimeter scale as previously described [11].

Determination of Minimum Inhibitory Concentration (MIC)

Minimum inhibitory concentration (MIC) of methanolic leaf extract was determined by a two-fold serial dilution method [13]. Briefly, the bacterial broth culture (to mid-log phase of absorbance at 600 nm reached at 0.4) was prepared as described above by inoculating bacterial strain into the nutrient broth media. The crude leaf extract of methanol were dissolved in Nutrient broth medium in an eppendorf tube to get a concentration of 20 mg/ml and further, serially diluted to achieve 10, 5, 2.5, 1.25, 0.625 and 0.312 mg/ml of concentrations. The 0.5 ml of bacterial broth culture of each tested bacteria was transferred to each tube. Thus, the total amount of solution in each eppendorf tube was 1 ml. The control tubes contain only 0.5 mL bacterial broth cultures with 0.5 ml nutrient broth media. The solutions of all eppendorf tubes were mixed properly by vortexing and incubated at 37 °C for 24 hours. After incubating for 24 hours, 100 μ L of solution from each eppendorf tube were spreaded over the nutrient-agar-media plate. The plates were incubated at 37 °C for 16 hours for bacterial growth and the number of colony was counted for MIC determination.

Results

Determination of Antibacterial Activity

The methanolic leaf extract (10 μ L of 20 mg/ml to each disc corresponding to 200 μ g/disc) of *Bixa orellana* L. significantly exhibited strong antibacterial effects against two Gram positive bacteria (*Bacillus subtilis*, *Sarcinalutea*) and five Gram negative bacteria (*Escherichia coli*, *Salmonella typhi*, *Shigella dysenteriae*, *Klebsiella pneumonia*, and *Proteus vulgaris*) with their respective diameters of inhibition zones of 9 to 14 mm (Figure 1). However, no antibacterial activity was observed against two Gram negative bacteria: *Xanthomonas campestris* and *Pseudomonas denitrificans* at the used concentration of 200 μ g/disc of plant extract. In this study, some commercial antibiotics were tested against the bacteria. However, the antibiotics that were used as positive control, showed higher antimicrobial activities than the plant leaf methanol extract against some bacteria. In addition, *Salmonella typhi* showed resistance against commercial antibiotics, but plant extract exhibited strong antibacterial effects against *Salmonella typhi* (Table 1).

Table 1: Antibacterial activity of methanol extract derived from the leaf of *Bixa orellana* L.

Bacteria mm	Zone of inhibition				
	Methanol extract ^a	Standard antibiotic ^b			
	Methanol	TC30	E15	GEN10	CIP5
<i>Bacillus subtilis</i>	10	16	20	25	23
<i>Sarcinalutea</i>	8	14	18	15	14
<i>Escherichia coli</i>	14.5	21	13	27	28
<i>Salmonella typhi</i>	14	-	-	-	-
<i>Shigella dysenteriae</i>	13.5	18	19	18	22
<i>Klebsiella pneumonia</i>	10	17	20	25	25
<i>Proteus vulgaris</i>	10	17	20	25	25
<i>Xanthomonas campestris</i>	nd	16	15	19	33
<i>Pseudomonas denitrificans</i>	nd	25	16	29	27

^a Diameter of inhibition zones of methanol extract including diameter of disc 6 mm (tested volume 10 μ L of 20 mg/ml to each disc corresponding to 200 μ g/disc).

^b Standard antibiotics : TC 30: Tetracycline (30 μ g/disc); E 15: Erythromycin (15 μ g/disc); GEN 10: Gentamicin (10 μ g/disc); CIP 5: Ciprofloxacin (5 μ g/disc). nd: No detection.

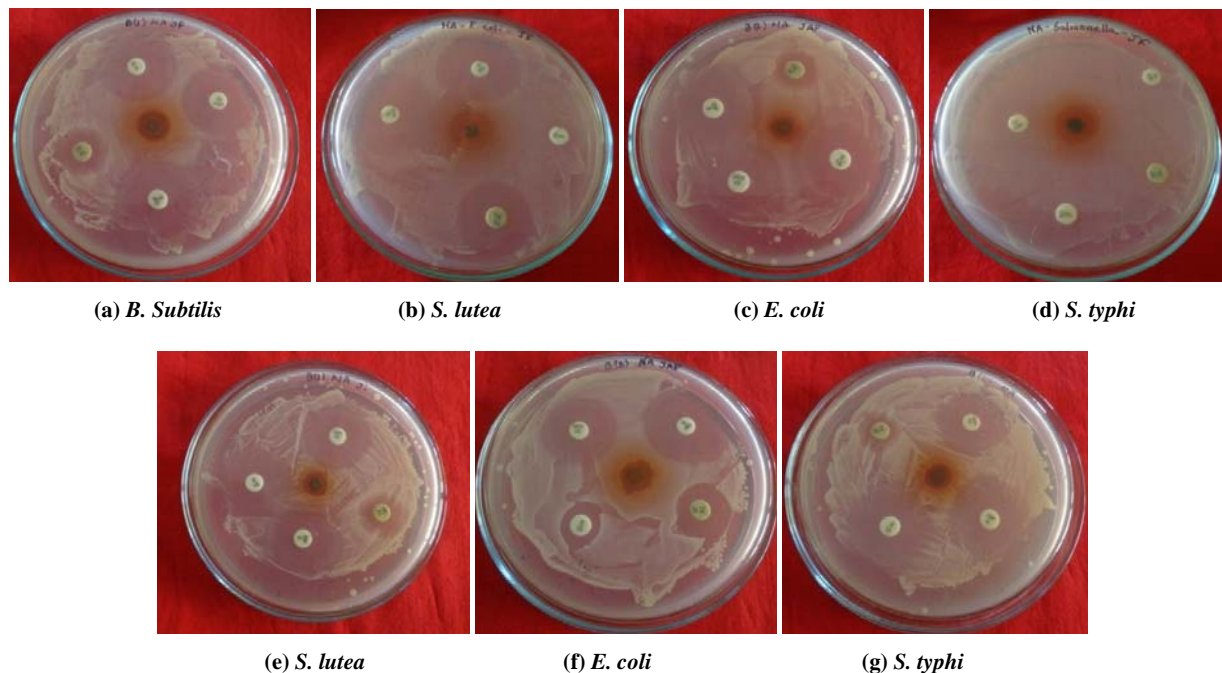


Fig 1: Antibacterial activity of Methanol extracts of *Bixa orellana* L. Leaf & commercial antibiotics. (a) *B. subtilis* with methanol extract & Gentamycin, Tetracycline, Ciprofloxacin, Erythromycin; (b) *S. lutea* with methanol extract & Gentamycin, Tetracycline, Ciprofloxacin, Erythromycin. (c) *E. coli* with methanol extract & Gentamycin, Tetracycline, Ciprofloxacin, Erythromycin; (d) *Salmonella typhi* with methanol extract & Gentamycin, Tetracycline, Ciprofloxacin, Erythromycin; (e) *S. dysenteriae* with methanol extract & Gentamycin, Tetracycline, Ciprofloxacin, Erythromycin (f) *K. pneumonia* with methanol extract & Gentamycin, Tetracycline, Ciprofloxacin, Erythromycin; (g) *P. vulgaris* with methanol extract & Gentamycin, Tetracycline, Ciprofloxacin, Erythromycin;

Minimum Inhibitory Concentration

The minimum inhibitory concentrations (MICs) defined as the lowest concentration of methanol extract that resulted in complete growth inhibition as no colony formation of the tested bacteria were found in the range of 5 to 10 mg/ml (Table 2). The organic extract displayed significantly

remarkable antibacterial activity against two Gram positive bacteria, *B. subtilis*, *S. lutea* and one Gram negative bacteria *S. dysenteriae* with their MIC value of 5 mg/ml, and four other Gram negative bacteria: *E. coli*, *S. typhi*, *K. pneumonia* and *P. vulgaris* with their MIC value of 10 mg/mL.

Table 2: Minimum inhibitory concentration of Methanol extracts derived from the leaf of *Bixa orellana* L. against different bacterial strain.

Bacteria(number of colonies)	Minimum inhibitory concentration (MIC)						
	Methanol extract concentration (mg/ml)						
	20	10	5	2.5	1.25	0.625	0.312
<i>B. subtilis</i>	0	0	0	4	24	32	86
<i>S. lutea</i>	0	0	0	3	35	66	103
<i>E. coli</i>	0	0	3	25	37	98	167
<i>S. typhi</i>	0	0	1	5	19	77	156
<i>S.dysenteriae</i>	0	0	0	4	48	87	198
<i>K. pneumonia</i>	0	0	6	34	59	84	224
<i>P. vulgaris</i>	0	0	2	4	46	98	196

Discussion

The methanol extract significantly exhibited strong antibacterial effects against two Gram positive bacteria (*Bacillus subtilis*, *Sarcinalutea*) and five Gram negative bacteria (*Escherichia coli*, *Salmonella typhi*, *Shigella dysenteriae*, *Klebsiella pneumonia*, and *Proteus vulgaris*) with their respective diameters of inhibition zones of 9 to 14 mm. This activity could be attributed to the presence of some bioactive phytochemical compounds such as vitamins (A, C, E, and K), carotenoids, terpenoids, flavonoids, phenols, unsaturated lactones, saponin, cyanogenic glycosides, glucosinolates and tannins in *Bixa orellana* L. plant and these finding are in agreement with the previous reports [14, 15, 16]. In recent years, several researchers have reported that the alkaloids, phenolics, triterpenoids, glycosides, tannins, etc. are

the major bioactive molecules from plant origins which have enormous potential to inhibit microbial pathogens [17, 18].

However, no antibacterial activity was observed against two Gram negative bacteria: *Xanthomonas campestris* and *Pseudomonas denitrificans* at the used concentration of 200 µg/disc of plant extract. It is often reported that Gram negative bacteria are more resistant to the plant-based organic extracts [19] because the hydrophilic cell wall structure of Gram negative bacteria is constituted essentially of a lipopolysaccharide (LPS) that blocks the penetration of hydrophobic oil and avoids the accumulation of organic extracts in target cell membrane [20]. This is the reason why Gram positive bacteria were found to be more sensitive to methanolic extract derived from *Bixa orellana* L. than were Gram negative bacteria.

In this study we observed that *Salmonella typhi* showed resistance against commercial antibiotics that were used as positive control, but plant extract exhibited strong antibacterial effects against *Salmonella typhi*. This is the most significant part of that study. This may be occurred due to the increased resistancy of *Salmonella typhi* against those commercial antibiotics. *Salmonella typhi* is a type of multi-drug resistance (MDR) strain. In all MDR strains so far examined, multiple resistances have been encoded by plasmids of the H1 incompatibility group^[21].

Conclusion

In this study, methanolic leaf extract of *Bixa orellana* L. demonstrated antibacterial activity against all the tested pathogenic bacteria. This activity could be attributed to the presence of major compounds (e.g., piperitonecarotenoids, terpenoids, flavonoids, phenols, unsaturated lactones, saponin, cyanogenic glycosides, glucosinolates and tannins). The result of this study suggests that *Bixa orellana* L. could be a source of new antibacterial agents in designing and developing new bio-drugs, employed in the treatment of infectious disease caused by multiple drug resistant bacteria. Toxicological tests for such extracts are necessary to avoid detrimental effects. The results obtained through *in vitro* test therefore, may not always translate to the same effects when administered into *in vivo* models. Pharmacological tests using *in vivo* models are therefore necessary to help confirm and further ascertain the efficacious properties of such extracts in living systems.

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