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In vitro evaluation of antibacterial activity of bioactive compound of *Bixa orellana* L. (Seed) against five important species of bacteria

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Abstract
In vitro evaluation of antibacterial activity of bioactive compound of *Bixa orellana* L. seed were evaluated against five pathogens viz., *Escherichia coli, Enterobacter aerogenes, Proteus vulgaris, Klebsiella pneumonia* and *Bacillus cereus* tested at 100 to 1000 ppm concentration. Maximum inhibition was observed in *E. coli,* and recorded 39.0mm inhibition at 1000ppm concentration, followed by *P. vulgaris* and recorded 36.0mm at 1000ppm concentration. *E. aerogenes* and *B. cereus* recorded 31.0mm and 26.0mm inhibition at 1000ppm concentration and least activity was observed in *K. pneumonia* and recorded 23.0mm at 1000ppm concentration,. Compared to standard antibiotic gentamycein at a recommended concentration of 25mg.

Keywords: *Bixa orellana,* bioactive compound, antibacterial activity, synthetic antibiotic

Introduction
The increasing prevalence of multidrug resistant strains of bacteria and the recent appearance of strains with reduced susceptibility to antibiotics raises the spectre of untreatable bacterial infections and adds urgency to the search for new infection-fighting strategies (Sieradski *et al*. 1999) [1]. Interest in plant-derived drugs has been increasing, mainly due to the current widespread belief that "green medicine" is safer and more dependable than costly synthetic drugs, many of which have adverse side effects. (Parekh, 2006) [4]. Many potent drugs including anti-malarial, anti-bacterial and anti-diabetic compounds have been purified from medicinal plants (Schmidt *et al.,* 2008) [3]. For centuries, plants have been used by indigenous people to produce medicines that were used to treat different kinds of ailments (Samie *et al.,* 2010) [2]. It contains many chemical compounds such as alkaloids, flavonoids, glycosides, phenols, resins, steroids, saponins, tannins and volatile oils which were deposited in their specific parts such as flowers, fruits, bark, leaves, root and seeds etc. (Digambar and Sahera, 2018) [14]. The antimicrobial properties of medicines from medicinal plants have been distinguished since ancient times. Plants characterize an excellent source of new antimicrobial molecules. Considering the ill effects of synthetic antibiotics, in the present study, bioactive compound isolated from the seeds of *B. orellana* L. belongs to family Bixaceae was evaluated for antibacterial activity against some important bacterial species in *in vitro* condition.

Materials and Methods
Plant material: Healthy seeds of *B. orellana* L. were washed with tap water thrice and two to three times with distilled water. The seeds were air dried at room temperature. Completely air dried seeds were powered and preserved until further use.

Extraction
Solvent extraction: Thoroughly washed seed of *B. orellana* were dried in shade for five days and then powdered with the help of Waring blender. 25 grams of shade dried powder was filled in the thimble and extracted with methanol in a Soxhlet extractor for 48 hours. Solvent extract was concentrated under reduced pressure. After complete evaporation, 1 gram of concentrated methanol extract was dissolved in 9 ml of methanol and used for antibacterial assay (Lalitha *et al*., 2011) [9].

Separation of bioactive compound by Thin Layer Chromatography (TLC)
Preparation of TLC plates and separation of bioactive compound: Five 20cm x 20cm glass plates were taken for coating with silica gel. Plates are thoroughly washed with detergent and water, rinse with distilled water and allow to drain. Plates were wiped with acetone soaked

tissue to remove grease and dirt. Plates were mounted on plate spreader and plates were clamped to provide an even spreading surface. 25 grams of silica gel adsorbant was mixed with 60 to 70ml of distilled water. The gap of the TLC applicator was adjusted to 0.25mm using feeler gauge provided. The applicator was placed on the end. Silica gel slurry was poured into spreader and with a single constant motion, the slurry was drawn along the plates. After spreading, the plates were incubated at 110° to 120°C overnight and cooled in a desiccator before use. On thin layer plates, gently mark the intended positions of samples with a clean pointed glass rod at one horizontal edge of the plate. The obtained concentrated Methanol extract was dissolved in 10 micro liter of their respective solvents and used for loading it into prepared TLC plates. All the loaded samples were eluted with methanol: chloroform extract In the ratio 9:1. After the elutent were run for more then 3/4th of the TLC plates, the plates were removed and examined under normal, short wavelength UV (254 nm) and long wavelength UV (366 nm) light in UV chamber. The obtained bands were divided into three fractions and each bands consists of three bands. R_f value of each band were calculated using the formulae Distance moved by compound / Distance moved by solvent system and used further for antibacterial activity (Sharma *et al*., 2009) [11].

Test organisms: Five pathogenic bacteria viz., *Escherichia coli, Enterobacter aerogenes, Proteus vulgaris, Klebsiella pneumonia and Bacillus cereus* were collected from research center, Pooja Bhagavat Memorial Mahajana P.G. Centre, K.R.S. Road, Metagalli, Mysore. The obtained cultures were sub-cultured on nutrient agar medium. After 24 hours of incubation at 37°C the cultures were preserved aseptically in lower temperature until further use.

Preparation of Inoculum: A loopful of all the test bacteria were taken and sub-cultured in test tube containing 10 ml of nutrient broth. The test tubes were incubated at 37°C for 24 hours. The broth was standardized using sterile normal saline to obtain a population of 10 cfu/ml.

Antibacterial assay

Agar Cup Diffusion Method: An overnight culture of *E.coli, E. aerogenes, P. vulgaris, K. pneumonia and B. cereus* were inoculated into petri plates containing nutrient agar medium. A sterile cork borer (5.0 mm diameter) was used to punch wells in the nutrient agar. Five wells were made in the petriplate containing media (One in center and four at the border), the agar plugs were removed with a sterilized wire loop. For each well 50 µl of different concentrations (100 to 1000 ppm) of the bioactive compound was added. The plates were incubated at 37°C for 24 hours and the zone of inhibition was measured in millimeter. The experiments were repeated for five times (Joshi, 1999) [10].

Structural elucidation of the Bioactive compound

The bioactive compound, was subjected to ^{1}H- NMR, ^{13}C-NMR analysis.

^{1}H- NMR data analysis: The ^{1}H- NMR spectrum of the bioactive compound was recorded on a Bruker AM 400 F (400 MHZ) NMR spectrometer using $CDCI_3$ as a solvent and TMS as internal standard. All chemical shift values were expressed in δ scale as s= singlet, d= doublet, t= triplet, m= multiplet (Al-Fatimi *et al*., 2006) [12].

^{13}C NMR analysis: The ^{13}C-NMR spectra were obtained on a Burker spectrometer AM 400(400 MHZ) with the solvent signal as internal reference (Al-Fatimi *et al*., 2006) [12].

Statistical Analysis

The data were subjected to Tukey's HSD analysis. Data on percentages were transformed to arcsine and analysis of variance (Anova) was carried out with transformed values. The means were compared for significance using Tukey's HSD (P=0.05).

Result

Determination of R_f value of the bioactive compound: from the observation of TLC, the R_f value of the bioactive compound was 2.0. The ^{13}C and ^{1}H NMR spectral analysis was made for the further elucidation for structural analysis of the bioactive compound (Figure 1).

Antibacterial assay: *Among the five bacteria tested, E.coli* recorded a maximum inhibition of 39.0mm in 1000ppm concentration. At 700ppm concentration it was recorded 36.0mm, 37.0mm at 800ppm, 38.0 mm at 900ppm respectively. *E.coli* was followed by *P.vulgaris* and recorded 30.0mm, 30.0mm, 31.0mm, 32.0mm, 33.0mm, 34.0mm and 36.0mm at 400, 500, 600, 700, 800, 900 and 1000ppm respectively. Moderate activity was observed in *E.aerogenes* and recorded 30.0 and 31.0mm at 900ppm and 1000ppm concentration. *B.cereus* recorded 25.0, 26.0 and 26.0mm at 800, 900 and 1000ppm concentration. Least activity was observed in *K.pneumonia* and recorded 22.0, 23.0 and 23.0mm inhibition at 800, 900 and 1000ppm concentration. Compared to synthetic antibiotic gentamycein at 25mg concentration, *E.coli* recorded 28.0mm, *E.aerogenes* recorded 26.0mm, *P.vulgaris* recorded 31.0mm, *B.cereus* recorded 24.0mm and *K.pneumonia* recorded 20.0mm inhibition respectively (Table 1).

Table 1: Antibacterial activity of bioactive compound of *Bixa orellana* L. (Seed) against five important species of bacteria

Bacteria	Concentration of the Bioactive compound										Gentamycin 25mg
	100 ppm	200 ppm	300 ppm	400 ppm	500 ppm	600 ppm	700 ppm	800 ppm	900 ppm	1000 ppm	
E.coli	23.0±0.0	26.0±0.0	30.0±0.0	31.0±0.0	33.0±0.0	34.0±0.0	36.0±0.0	37.0±0.0	38.0±0.1	39.0±0.0	28.0±0.0
E.aerogenes	19.0±0.1	20.0±0.1	21.0±0.0	23.0±0.0	23.0±0.0	24.0±0.1	24.0±0.0	26.0±0.0	30.0±0.1	31.0±0.0	26.0±0.0
P.vulgaris	20.0±0.0	26.0±0.0	28.0±0.0	30.0±0.0	30.0±0.0	31.0±0.1	32.0±0.0	33.0±0.0	34.0±0.1	36.0±0.0	31.0±0.0
B.cereus	13.0±0.0	17.0±0.0	19.0±0.1	20.0±0.0	21.0±0.1	22.0±0.1	22.0±0.1	25.0±0.0	26.0±0.0	26.0±0.1	24.0±0.0
K.pneumonia	9.0±0.1	16.0±0.0	18.0±0.0	19.0±0.0	19.0±0.0	20.0±0.0	21.0±0.0	22.0±0.1	23.0±0.0	23.0±0.1	20.0±0.0

- Values are the mean of five replicates, ±standard error.
- The means followed by the same letter (s) are not significantly different at P 0.05 when subjected to Tukey's HSD.
- Pattern of percentage inhibition increase is not uniform for all the microorganisms

Structural elucidation of the bioactive compound

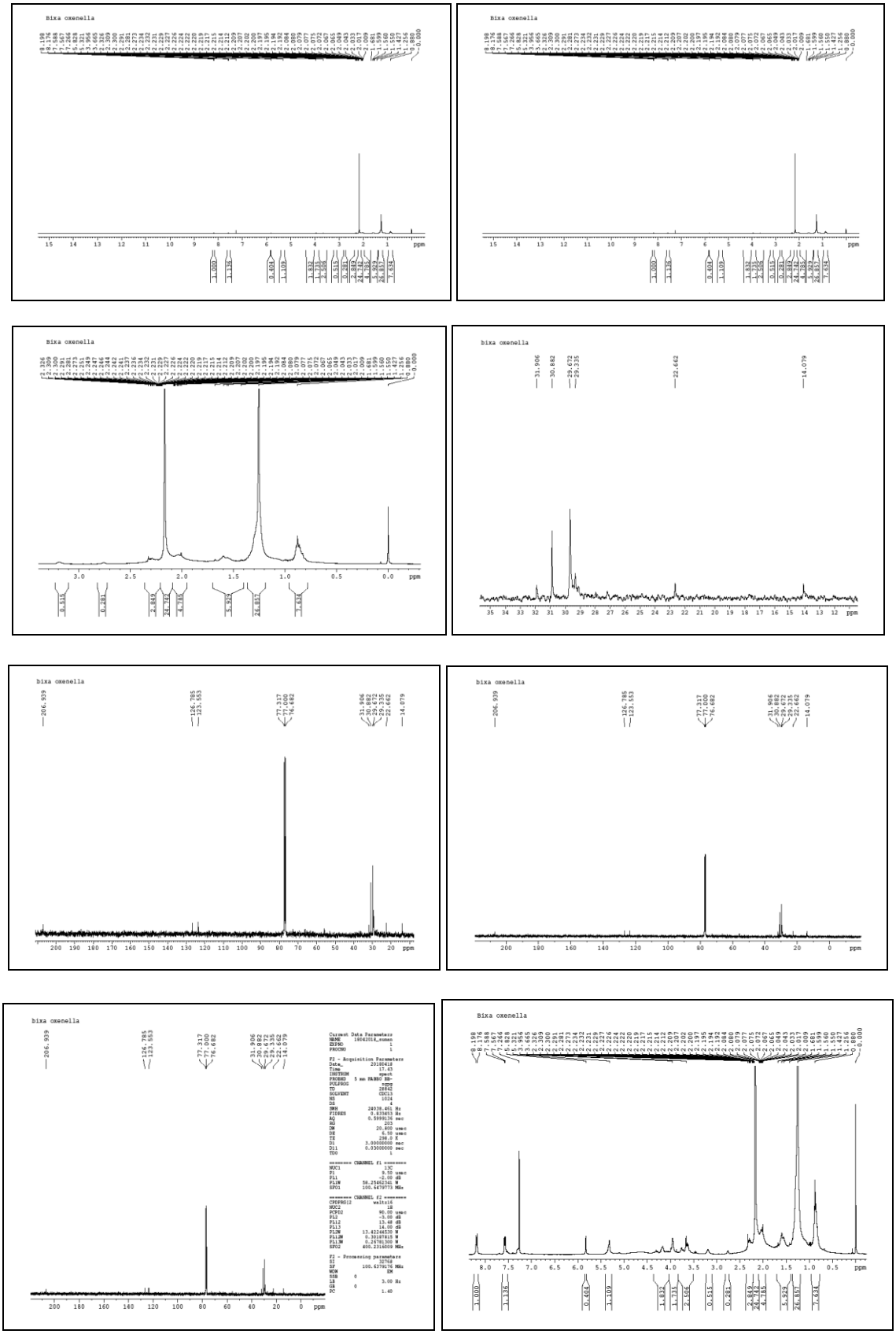

Fig 1: ^{13}C and ^{1}H NMR spectra of Bioactive compound isolated from the seeds of *Bixa orellana* L.

Discussion

In the present time multiple drug resistance in microbial pathogens become a serious health problem to humankind worldwide (Peng *et al.*, 2006) [7]. It is aroused due to indiscriminate and repetitive use of antimicrobial drugs by inadequate disease treatment (Shariff, 2001) [8]. Various medicinal plants have been used for years in daily life to treat disease all over the world. The use of traditional plant extracts as well as other alternative forms of medical treatments have been getting momentum since the 1990s (Abbas Ali, 2008: Cowan, 1999) [13]. It has been observed that, bioactive compound of seeds of *Bixa orellana* showed a promising

result in managing all the five test pathogens tested from 100 to 1000 ppm concentration. Hence seeds of *B. orellana* is an alternative medicinal source for the management of pathogenic bacteria.

Conclusion

From the above result, it can be concluded that, further investigation is necessary to elucidate the structure of the bioactive compound isolated from the seeds of *B.orellana* which will be needed for future drug formulation.

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