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## ***In vitro* Antibacterial Activity of n-Hexane Fraction of Methanolic Extract of *Plumeria rubra* L. (Apocynaceae) Stem Bark**

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### **ABSTRACT**

Medicinal plants serve as sources of valuable compounds with therapeutic potential. *Plumeria rubra* L. (Apocynaceae) is a medicinally important tree which has been reported as ethnomedicinal cure of different ailments. In the current investigation, n-hexane fraction of crude methanolic extract of *P. rubra* stem bark was investigated against four ATCC (American type culture collection) bacterial strains *Staphylococcus aureus* (ATCC 25923), *Enterobacter cloacae* (ATCC 13047), *Pseudomonas aeruginosa* (ATCC 27853) and *Serratia marcescens* (ATCC 13880). Inhibitions of the plant extract against all the four microorganisms were tested by both agar-diffusion assay and broth microdilution method. The n-hexane fraction of crude methanolic extract of *P. rubra* stem bark showed MICs of 13.5, 11.8, 8.5 and 16.9 mg mL<sup>-1</sup> and induced a maximum of 91.53, 92.84, 94.69 and 85.29% growth inhibition against *S. aureus* (ATCC 25923), *E. cloacae* (ATCC 13047), *P. aeruginosa* (ATCC 27853) and *S. marcescens* (ATCC 13880), respectively. The active plant extract in this study showed significant antibacterial activities against all the human pathogenic strains, adding credence to the ethnomedicinal uses of the plant, as well as, suggesting towards its specific use against the tested microorganisms.

**Key words:** *Plumeria rubra*, n-hexane, minimum inhibitory concentration, American type culture collection, colony forming units

### **INTRODUCTION**

Plants and plant derived medicine play a crucial role in the popular herbal medicine as well as other alternative and complementary system of medicine. A huge percentage of the world's population partially or entirely depend on botanicals as a part of their day to day health care. The ethnobotanical wealth of Indian subcontinent is one of the biggest sources of herb based drugs with potential to be included in drug discovery programmes (Dey and De, 2012a,b). Crude plant extracts are used as ethnomedicine to treat different ailments. Herbs and herbal medicines may substitute the conventional systems of medicine in future due to lesser side effects and cost effectiveness. Although, sometimes the crude plant extracts are reported to possess greater efficacy than the isolated biomolecules, plants are known to produce a number of bio active compounds with potent pharmacological significance. Many plant species have been reported to possess antimicrobial activities (Bonjar *et al.*, 2004; Belboukhari and Cheriti, 2005; Thakurta *et al.*, 2007; Lokhande *et al.*, 2007; Pieme *et al.*, 2008; Abukakar *et al.*, 2008; Sunilson *et al.*, 2009;

Brito-Argaez *et al.*, 2009; Krishnan *et al.*, 2010; Khanahmadi *et al.*, 2010; Dey, 2011; Mishra and Mishra, 2011; Dey and De, 2010, 2011; Jazani *et al.*, 2011; Sundaram *et al.*, 2011; Viveros-Valdez *et al.*, 2011; Mukherjee *et al.*, 2011). Although most of the experiments are performed *in vitro* and/or *in vivo*, the experiments must be extended to the next level of clinical trial to include them in drug discovery programmes (Dey and De, 2011).

*Plumeria rubra* L. (*Frangipani*, Temple tree), a member of the Apocynaceae family, is native to central and southern parts of America. Fragrant pink, white and yellow flowers are the characteristic feature of the deciduous plant. It is well acclimatized to grow as a popular garden and ornamental plant in the tropical and subtropical parts of the globe. The plant serves as an important ethnobotanical remedy as a part of the traditional healing system of different ethnic groups (Gopi *et al.*, 2011).

*P. rubra* is used in ulcer, inflammation, bronchitis, fever (Baghel *et al.*, 2010; Zaheer *et al.*, 2010), stomachache (Ramana, 2008), eye cleaning (Ruiz-Teran *et al.*, 2008), diarrhoea (Basavaraju *et al.*, 2009), dysentery (Sen *et al.*, 2011), wound healing (Rajakumar and Shivanna, 2010), respiratory disorder (asthma) (Patil *et al.*, 2008), birth control (Tiwari *et al.*, 1982; Kalita *et al.*, 2011), rheumatism (Basavaraju *et al.*, 2009), cancer (Kuete and Efferth, 2011) and snakebite (ethnoveterinary) (Deshmukh *et al.*, 2011) in different systems of medicine. The plant has also been investigated for antibacterial (Hamburger *et al.*, 1991; Egwaikhide *et al.*, 2009; Kuigoua *et al.*, 2010), antifungal (Kuigoua *et al.*, 2010; Souza *et al.*, 2011; Gaitan *et al.*, 2011), antialgal (Kuigoua *et al.*, 2010), larvicidal (Ramos *et al.*, 2009), molluscicidal (Hamburger *et al.*, 1991), piscicidal (Joshi and Joshi, 2006), antioxidant and free radical scavenging activities (Ruiz-Teran *et al.*, 2008), proteolytic (De Freitas *et al.*, 2010), cytotoxic (Kardono *et al.*, 1990; Hamburger *et al.*, 1991) and anti HIV (Tan *et al.*, 1991) properties. The present investigation demonstrates the *in vitro* antibacterial efficacy of n-hexane fraction of the crude methanolic extract of *P. rubra* stem bark against some potent human pathogenic bacterial strains.

## MATERIALS AND METHODS

**Plant material:** Stem bark was harvested from a 10 years old plant during the month of June, 2011 from Baruipur, South 24 Parganas, West Bengal, India. The plant was identified by a Taxonomist and a voucher specimen was preserved in Molecular Biology Laboratory, Department of Zoology and Molecular Biology and Genetics, Presidency University.

**Bacterial strains:** The four ATCC bacterial strains used in the study were *Staphylococcus aureus* (ATCC 25923), *Enterobacter cloacae* (ATCC 13047), *Pseudomonas aeruginosa* (ATCC 27853) and *Serratia marcescens* (ATCC 13880). These strains were cultured on nutrient agar (Himedia, India). For the purpose of storage, grown bacteria were then picked from the medium and maintained in nutrient agar stab at room temperature and in nutrient broth containing 10% glycerol (Merck, Germany) at -20°C until testing.

**Extraction:** The stem bark segments were cleaned thoroughly by sterile distilled water and air dried at 29°C for 15 days and grinded to powder by a mixer grinder. One kg of powder was soaked thrice in methanol at room temperature (29°C) for 15 days. The following steps of methanol extraction and n-hexane fractionation were done on the basis of a previous report (Mukherjee *et al.*, 2011). The various concentrations of n-hexane fraction were prepared by dissolving the requisite amount of residue to the solvent.

**Antimicrobial susceptibility testing:** Antibacterial activity of the extract was determined by agar-diffusion assay (Reeves, 1989) with the following modifications. Bacterial strains were first grown in Mueller Hinton Broth (MHB) under shaking condition for 4 h at 37°C and after the incubation period, 1 mL of culture were spread on Mueller Hinton Agar (MHA) plate. In the inoculated MHA plate, wells were made by using sterile 6 mm cork borer. The wells were filled with 200 µL of the plants extracts (re-suspended in n-hexane) and blanks (n-hexane). The concentrations of extract employed were 25, 50, 100 and 200 mg mL<sup>-1</sup>. Tetracycline (150 µg mL<sup>-1</sup>, 200 µL) was used as antibacterial positive control. Zone diameter was measured after 24 h incubation at 37°C.

**Determination of minimum inhibitory concentration (MIC):** MIC of the extract was assessed using the broth microdilution method (Mukherjee *et al.*, 2011) following the recommendation by the National Committee for Clinical Laboratory Standards (NCCLS, 1997, 1999, 2002). n-hexane was used as negative control. Minimum inhibitory concentrations (MIC) were read visually. After three independent trials, the lowest concentration which did not show any growth of the tested microorganism, was interpreted as the MIC.

**Statistical analysis:** Calculations were carried out in triplicate with their mean values and standard error following the formula of Pagano and Gauvreau (2000). Values are expressed as Mean±S.D. Statistical significance was determined using Student's t-test. Values with p<0.05 were considered significance. Preparation of tables and graphs were done by Microsoft Office Excel (2007).

## RESULTS

**In-vitro antibacterial activity of *P. rubra* stem bark extract:** Strains of four bacterial cells, *S. aureus* (ATCC 25923), *E. cloacae* (ATCC 13047), *P. aeruginosa* (ATCC 27853) and *S. marcescens* (ATCC 13880) were tested to evaluate the antibacterial activity of the n-hexane fraction of crude methanolic extract of *P. rubra* stem bark.

However, the n-hexane fraction of crude methanolic extract of stem of *P. rubra* stem bark showed inhibitory activities against all the strains by agar-diffusion assay with significance (p<0.05) (Table 1). At the highest of the concentrations tested, (200 mg mL<sup>-1</sup>), the extract showed maximum activity against *S. aureus* (17.7±0.8 mm) and minimum activity against *S. marcescens* (12.7±0.6 mm). Antibacterial activity was expressed in terms of diameter of zone of inhibition (Mean±SD n = 3). p<0.05 compared to control (n-hexane) is considered significant.

Table 1: Antibacterial activity of *P. rubra* stem bark extract on four bacterial strains

Bacterial strain	Zone of inhibition diameter (mm)				
	n-hexane fraction of methanolic extract (200 µL well <sup>-1</sup> )				Control (n-hexane)
	200 mg mL <sup>-1</sup>	100 mg mL <sup>-1</sup>	50 mg mL <sup>-1</sup>	25 mg mL <sup>-1</sup>	
<i>S. aureus</i>	17.7±0.8	14.8±0.4	12.8±1.3	9.8±0.9	0.00
<i>E. cloacae</i>	15.8±0.8	12.9±0.9	10.7±0.8	8.2±0.7	0.00
<i>P. aeruginosa</i>	17.4±0.7	14.3±1.2	12.6±0.6	9.4±0.9	0.00
<i>S. marcescens</i>	12.7±0.6	10.1±0.5	8.8±0.7	6.4±0.6	0.00

Antibacterial activity was expressed in terms of diameter of zone of inhibition (Mean±SD n = 3). p<0.05 compared to control (n-hexane) is considered significant

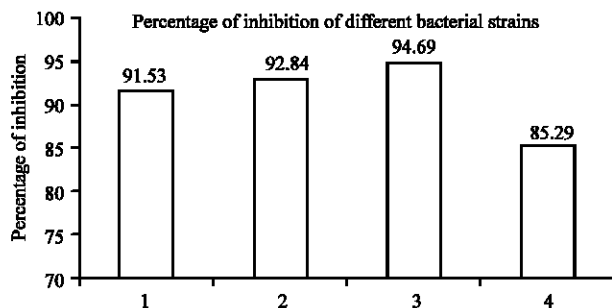


Fig. 1: The percentage of inhibition of different bacterial strains by n-hexane fraction of the crude methanolic extract of *P. rubra* stem bark. 1: *S. aureus* (ATCC 25923) 2: *E. cloacae* (ATCC 13047) 3: *P. aeruginosa* (ATCC 27853) and 4: *S. marcescens* (ATCC 13880)

The n-hexane fraction of crude methanolic extract of stem of *P. rubra* stem bark induced a maximum of 91.53, 92.84, 94.69 and 85.29% growth inhibition against *S. aureus* (ATCC 25923), *E. cloacae* (ATCC 13047), *P. aeruginosa* (ATCC 27853) and *S. marcescens* (ATCC 13880), respectively (Fig. 1).

## DISCUSSION

In some previous experiments, the plant was reported for antimicrobial activity. Leaf powder of the plant extracted in ethanol, chloroform, ethyl acetate and water was tested against *Staphylococcus epidermidis* (MTCC 3615) and *Escherichia coli* (MTCC 118) by disc diffusion method and some of the extracts has exhibited potency against the bacteria (Baghel *et al.*, 2010). Essential oil isolated from the flowers of *P. rubra* along with two other *Plumeria* sp. was investigated by agar diffusion method against a number of bacterial and fungal species such as *Escherichia coli*, *Staphylococcus aureus*, *Bacillus cereus*, *Candida albicans*, *C. humicola*, *Trichophyton mentagrophytes*, *T. rubrum* and *Microsporium canis*. *P. rubra* was found to be effective against four of the bacterial strains (Sulaiman *et al.*, 2008). Interestingly, the stem bark, with which the present investigation was performed, has been reported to produce a number of compounds with antimicrobial activity (Kuigoua *et al.*, 2010). Heartwood of the plant has yielded plumericin and isoplumericin exhibiting antibacterial activity (Hamburger *et al.*, 1991). Moreover, the plant has been investigated against subcutaneous antifungal infections (Gaitan *et al.*, 2011). In another experiment, laticifer proteins of the species were tested against plant pathogens (Souza *et al.*, 2011). Stigmasterol, beta-sitosterol, lupeol and its acetate, arjunolic acid, ursolic acid, oleanolic acid, beta-amyrin acetate, betulinic acid, P-E-coumaric acid, 2,6-dimethoxy-P-benzoquinone, scopoletin and iridoids were isolated from the stem bark (Kuigoua *et al.*, 2010). Most of these compounds are reported to possess antibacterial and/or antifungal efficacy. Antimicrobial activity of ursolic acid (Collins and Charles, 1987), oleanolic acid (Horiuchi *et al.*, 2007), arjunolic acid (Hemalatha *et al.*, 2010), lupeol (Siddique and Saleem, 2011), beta-sitosterol, lupeol acetate, beta-amyrin (Tahany *et al.*, 2010), betulinic acid (Yoggeswari and Sriram, 2005), iridoids (Yang *et al.*, 2006), pcoumaric acid (Herald and Davidson, 1983), 2,6-dimethoxy-P-benzoquinone (Nishina *et al.*, 1991), scopoletin (Carpinella *et al.*, 2005) and stigmasterol (Kabouche *et al.*, 2005) is reported. The results derived from the present investigation could be attributed to the antimicrobial compounds present in the stem bark of the medicinal plant. Presence of diverse

groups of antimicrobial biomolecules in different parts of the plant including the stem bark also supports the results of the present investigation.

The study has two fold significance. First of all, a positive correlation is being established between the ethnomedicinal uses of the plant and the present antibacterial investigation. The human pathogenic bacterial strains used in the present investigation occur in skin, soft tissues, wound, respiratory tracts, urinary tracts, gastrointestinal tract, endovascular system etc. causing an array of diseases such as pneumonia, severe skin infections, bacteremia, sepsis, meningitis, endocarditis, osteomyelitis, toxic shock syndrome, lower respiratory tract infections, ophthalmic infections, intra-abdominal and gastrointestinal infections, septic arthritis, urinary tract infections, wound infections, meningitis and cerebral abscesses. The species, on the other hand, is traditionally used in ulcer, inflammation, respiratory disorder (asthma), bronchitis, stomachache, eye cleaning, diarrhoea, dysentery, wound healing, rheumatism etc. Ethnomedicinal efficacy of the plant species against respiratory disorders, gastrointestinal problems and skin and wound infection may have been contributed by the antimicrobial constituents of the plant. As the plant extract have acted as a potent inhibitor of some of the very common human pathogenic bacterial strains, isolation of the active biomolecules may lead to the discovery of novel remedy for some of the serious and life threatening diseases causing human morbidity and mortality.

Secondly, prolonged use of conventional synthetic antibiotics develops drug resistance in different bacterial strains. In addition to that, adverse side effects and high cost are among the other negative aspects of using them especially in the underprivileged parts of the world. Plant derived natural substances and natural compound based molecules may serve as alternative treatment of different pathogenic diseases, where drug resistance, side effects and cost effectiveness are the major points of concern.

## CONCLUSION

The human pathogenic bacterial strains used in the antibacterial experiments are known to cause some mild to severe infections which sometimes result into mortality. These stains, however, were found to be significantly inhibited by n-hexane fraction of the methanolic extract of stem bark powder of *P. rubra*. The study provides evidence for the plant's use in certain folk practices in the treatment of different ailments. Although, the *in vitro* methods of pharmacological testing of crude herbal preparations serve as a useful tool to begin with, positive results from which may lead to fractionation and purification of active compounds. Further *in vivo* experiments and clinical trials are required in order to include it in drug discovery programs.

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