International Journal of Biomedical and Pharmaceutical Sciences ©2007 Global Science Books

Antibacterial Activity of Stem Bark Constituents of *Polyalthia cerasoides* (Roxb.) Bedd.

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ABSTRACT

Stem bark extract of *Polyalthia cerasoides* was screened against 24 clinical isolated strains from different infectious sources: Gram-negative *Pseudomonas aeruginosa* and *Klebsiella pneumonia* and Gram-positive *Staphylococcus aureus*. Significant minimum inhibitory zones were recorded with the ethyl acetate fraction (20.12 ± 0.29, 20.00 ± 0.11 and 18.35 ± 0.20 mm diameter against *K. pneumonia*, *P. aeruginosa* and *S. aureus*, respectively) more than with the dichloromethane fraction (15.07 ± 0.11, 13.52 ± 0.22 and 14.67 ± 0.25 mm diameter against *K. pneumonia*, *P. aerugenosae* and *S. aureus*, respectively). Both fractions showed activity against standard ATCC and MTCC strains. Ciprofloxacin was used as standard.

Keywords: Annonaceae, antibacterial resistance, berberin, clinical isolates, medicinal plants
Abbreviations: ANOVA, Analysis of Variance; **ATCC**, American Type Cell Culture; **DCM**, dichloromethane; **DMSO**, dimethylsulphoxide; **EA**, ethyl acetate; **MTCC**, Microbial Type Culture Collection; **MIC**, minimum inhibition concentration

INTRODUCTION

The increased prevalence of antibiotic resistant bacteria due to the extensive use of antibiotics may render the current antimicrobial agents insufficient to control bacterial diseases (Cowan 1999). In most countries popular herbal medicines are increasingly used as remedies for many infectious diseases. Plants have provided a source of inspiration for novel drug compounds, as plant-derived medicines have made large contributions to human health and well-being. The acceptance of traditional medicine as an alternative form of health care and the development of microbial resistance to available antibiotics has led many researchers to investigate the antimicrobial activity of medicinal plants (Benkeblia 2004).

Polyalthia cerasoides (Roxb.) Bedd. (*Annonaceae*; **Fig. 1**) is a medium sized tree distributed in almost all forests of the Deccan region, India up to 3000 ft (Gamble 1935). Tribal people of Tamil Nadu and Andhra Pradesh use the fruits as food (Padma *et al*. 2001). In a previous study on *P. cerasoides* several prenylated benzopyran derivatives inhibited the mitochondrial respiratory chain (González *et al*. 1995, 1996; Zafra-Polo *et al*. 1996) and two oxoprotoberberine alkaloids (González *et al*. 1997) were isolated from the dichloromethane fraction and ethyl acetate fraction, respectively of the methanol extract of stem bark. Previous studies indicate that oxoprotoberberine alkaloids have certain properties, e.g. antibacterial (Hopp *et al*. 1976; Cowan 1999), Acetylcholinesterase inhibitor induces differentiation in neural cells, lowers cholesterol and stimulates insulin secretion (Zhu and Qian 2006). Pharmacological studies confirmed stem bark constituent of *Polyalthia cerasoides* could reduce brain stress (Padma *et al*. 2001).

Plants produce a great diversity of substances that could be active in many fields of medicine. Now some natural products have been approved as new antibacterial drugs, but there is an urgent need to identify novel substances active towards pathogens with resistance (Recio *et al*. 1989; Cragg *et al*. 1997). It is important to know the constituents of *P.*

Fig. 1 Photographs of *Polyalthia cerasoides* in natural habitat. (**A**) leaves (**B**) stem bark.

Received: 7 April, 2007. Accepted: 15 August, 2007.

Original Research Paper

cerasoides, since the plants belonging to this genera show remarkable antibacterial (George *et al*. 2002), antifungal (de Boer *et al*. 2005; Marthanda *et al*. 2005), anti HIV (Li *et al*. 1993) and antimalarial (Somdej *et al*. 2002) properties. However, the antibacterial potency of the constituent from the extract of *P*. cerasoides have not been investigated so far. Based on these findings we tested various fractions of alcohol extracts of *P*. *cerasoides* stem bark for their antibacterial potency.

MATERIALS AND METHODS

Plant material

P. *cerasoides* stem bark was collected in September 2006, from the Kuvempu University forest and authenticated through the Department of Biotechnology, Kuvempu University with the deposit of a voucher specimen (SNPS-015/2005-2006).

Extraction and fractionation

P. *cerasoides* stem bark was dried for 1 week at room temperature and reduced to coarse powder. 100 g of powder was extracted with 500 ml of methanol for 48 h. The extract was concentrated by a rotary evaporator (Büchi, Flawil, Switzerland), then further partitioned between water and dichloromethane (Fraction DCM) and then between water and ethyl acetate (fraction EA). Then both of these fractions were subjected to antimicrobial investigation.

In vitro antibacterial activity

The antibacterial activity of dichloromethane and chloroform fractions of methanol extract were screened by the agar well diffusion method (Mohsen Abolhassani 200)) against 24 clinical isolates of each of eight bacterial strains belonging to Gram-positive *Staphylococcus aureus* and Gram-negative *Klebsiella pneumonia*, and *Pseudomonas aeruginosa* (**Table 1**). The bacterial strains used for screening antimicrobial activity were collected from different infectious statuses of patients who had not taken any antibacterial drugs for at least two weeks with the help of an authorized physician, in the district health center of Gulberga, Karnataka State, India. The clinical isolates were identified following a standard method (Cown *et al*. 1993). The bacterial suspensions were diluted in 10-1 to 10-8 phosphate buffered saline. Samples were homogenized and then loaded in six aliquots of 20 µL each onto nutrient agar plates (agar 15 g/L, beef extract 1 g/L, peptone 5 g/L, NaCl 5 g/L, yeast extract 2 g/L; diameter 55 mm, final pH 7.0 ± 0.2). The plates were incubated for 24 h at 37°C and counting was done on plates containing 50 to 100 colonies. The activity was screened comparatively with reference ATCC strains (*Pseudomonas aeruginosa*: ATCC-20852; *Staphylococcus aureus*: ATCC 29737) and MTCC strain (*Klebsiella pneumonia*: MTCC-618). The fluoroquinolone antibiotic Ciprofloxacin (BioChemika, ≥98.0% (HPLC) (Fluka)) was used as the standard (50 µg/100 µL of sterilized distilled water) concomitantly with the test samples. The minimal inhibitory concentrations (MIC) of both the fractions were determined by micro dilution techniques in nutrient broth, according to the National Committee for Clinical Laboratory Standard, USA guidelines (Takemura *et al*. 1996). The inoculates were prepared in the same medium at a density adjusted to a 0.5 McFarland turbidity standard colony forming units and diluted to 1:10 ratio for the broth micro dilution procedure. The microtiter plates were incubated at 37°C and MIC was determined after 24 h of incubation.

Table 1 Profile of the clinical strains used for antibacterial activity.

Clinical strains	Clinical condition	Source
K. pneumonia		
Kp1	Pneumonia	Mucus
Kp2	Gram negative Follicullitis	Stipules
Kp3	Burns	Pus
Kp4	UTI	Urine
Kp5	Septicemia	Sputum
Kp6	Cross infections in UTI	Urine
Kp7	Abscess in immunodeficiency	Wounds
Kp8	Upper UTI	Urine
P. aeruginosa		
Pa1	Bronchitis	Wounds
Pa2	Otitis media	Pus
Pa3	Burns	Sputum
Pa4 and Pa5	Upper UTI	Stool
Pa6	Food poisoning	Hospital effluent
Pa7	Cross infections in UTI	Hospital effluent
Pa8	Septicemia	Old Wounds
S. aureus		
Sa1	Abscess in immunodeficiency	Wounds
Sa2	Burns	Pus
Sa3	Septicemia	Old Wounds
Sa4	Food poisoning	Stool
Sa5	Burns	Pus
Sa6 and Sa7	Unknown	Hospital effluent
Sa8	Abscess in immunodeficiency	Sputum

A sensitive radial diffusion technique was used for the assessment of antibacterial activity of the test samples. Sterilized nutrient agar medium was poured into sterilized Petri dishes. Nutrient broth containing 100 µl of 24 h incubated cultures of the respective clinical isolates and the ATCC and MTCC strains were spread separately on the agar medium. Wells were created using a stainless steel sterilized cork borer under aseptic conditions. Fractions and standard at 50 µg/100 µl were loaded to wells. The plates were incubated for 24 h at 37°C and the diameter of the zone of complete inhibition of the bacteria was measured around each well and readings were recorded in mm. The results of these experiments are expressed as mean ± SE of six replicates in each test. The data were evaluated by one-way ANOVA followed by Tukey's pairwise comparison test and the results were considered significant when $p<0.05$.

RESULTS AND DISCUSSION

All the strains of *K. pneumoniae* were isolated from different infectious statuses of patients. The EA fraction was most effective in controlling growth of these clinical and standard MTCC strain (**Table 2**). The maximum zone of inhibition of the EA fraction was 20.12 ± 0.29 recorded against the strain isolated from the pus of burn wounds. The standard, ciprofloxacin also showed a maximum zone of inhibition (21.07 ± 0.43) against the same strain. The DCM fraction exhibited maximum inhibitory activity of only 14.30 ± 0.17 against *K. pneumoniae* isolated from sputum. Among the zone of inhibition of DCM fraction and EA fraction, the EA fraction was found to be nearer to the zone of inhibition of standard ciprofloxacin in most of the isolated and standard strains.

The EA fraction was also a potent inhibitor for the growth of Gram-negative *P*. *aeruginosa*. The maximum zone of inhibition (20.00 ± 0.11) was observed with the strain isolated from hospital effluent. Against other strains of *P. aeruginosa*, the EA fraction showed a moderate zone of inhibition whereas standard drug ciprofloxacin recorded a maximum zone of inhibition of 21.17 ± 0.26 against the strain isolated from stool. The DCM fraction recorded a 13.52 ± 0.22 mm maximum zone of inhibition against bacteria isolated from hospital effluent. Towards other strains, the DCM fraction showed very little zone of inhibition compared to both the EA fraction and standard ciprofloxacin. The results are shown in **Table 3**.

Among the different clinical strains of *S. aureus* tested for antibacterial activity, the pathogen isolated from urinary track infection is more susceptible to the EA fraction compared to other pathogens, recording a maximum of 18.35 ± 0.20 mm zone of inhibition. The remaining strains were also more susceptible to the EA fraction than the DCM fraction. Thus zone of inhibition exhibited by the EA fraction against different clinical strains and the reference ATCC strain of *S. aureus* were found to be close to the standard ciprofloxacin in terms of effect. DCM fraction also showed a 14.47 ± 0.18 mm zone of inhibition against the reference and a maximum of 14.67 ± 0.25 mm against pathogens isolated from wounds. The EA fraction showed significantly

Table 2 Antibacterial activity of dichloromethane and ethyl acetate fractions against *Klebsiella pneumoniae*.

Bacterial strains tested	**Zone of inhibition (in mm)**		
Klebsiella pneumoniae	**DCM**	**EA**	**Reference drug (Ciprofloxacin)**
MTCC-618	12.40 ± 0.26 d	19.88 ± 0.42 ab	19.90 ± 0.13 d
Kp-1	11.47 ± 0.22 e	14.15 ± 0.41 gh	20.25 ± 0.28 bc
Kp-2	14.28 ± 0.18 b	15.67 ± 0.31 f	20.32 ± 0.30 bc
Kp-3	10.88 ± 0.13 f	20.12 ± 0.29 a	21.07 ± 0.43 a
Kp-4	14.05 ± 0.08 bc	18.02 ± 0.24 c	19.15 ± 0.24 dc
Kp-5	14.30 ± 0.17 b	15.98 ± 0.25 b	20.48 ± 0.25 b
Kp-6	15.07 ± 0.11 a	17.60 ± 0.18 d	20.17 ± 0.23 bc
Kp-7	12.20 ± 0.18 d	16.20 ± 0.24 e	19.45 ± 0.30 d
Kp-8	14.20 ± 0.16 b	14.68 ± 0.20 g	20.63 ± 0.26 b
F-Value	73.5	53.0	4.5

Kp = *Klebsiella pneumoniae*
The value of each constituents consisted of ± S.D. of six replicates.
The F-value is significantly different when $p<0.05\%$.
In each column the mean value with different alphabetical letters are significantly different

Table 3 Antibacterial activity of dichloromethane and ethyl acetate fractions against *Pseudomonas aeruginosa*.

Bacterial strains tested	**Zone of inhibition (in mm)**		
Pseudomonas aeruginosa	**DCM**	**EA**	**Reference drug (Ciprofloxacin)**
ATCC-	10.20 ± 0.12 e	18.17 ± 0.08 bc	18.68 ± 0.26 f
Pa-1	10.15 ± 0.13 ef	16.62 ± 0.14 d	20.27 ± 0.26 bc
Pa-2	12.82 ± 0.12 b	18.73 ± 0.30 b	20.22 ± 0.23 bc
Pa-3	11.97 ± 0.15 d	18.80 ± 0.26 b	19.07 ± 0.20 e
Pa-4	12.92 ± 0.24 b	16.97 ± 0.21 d	21.17 ± 0.26 a
Pa-5	10.93 ± 0.17 e	18.27 ± 0.11 bc	18.93 ± 0.30 f
Pa-6	12.90 ± 0.18 b	20.00 ± 0.11 a	20.72 ± 0.22 b
Pa-7	13.52 ± 0.22 a	16.80 ± 0.19 d	20.65 ± 0.17 b
Pa-8	12.72 ± 0.15 b	18.52 ± 0.16 b	19.85 ± 0.15 cd
F-value	56.4	34.9	14.0

Pa = *Pseudomonas aeruginosa*
The value of each constituents consisted of ± S.D. of six replicates.
The F-value is significantly different when $p<0.05\%$.
In each column the mean value with different alphabetical letters are significantly different

Table 4 Antibacterial activity of dichloromethane and ethyl acetate fractions against *Staphylococcus aureus*.

Bacterial strains tested	**Zone of inhibition (in mm)**		
Staphylococcus aureus	**DCM**	**EA**	**Reference drug (Ciprofloxacin)**
ATCC-	14.47 ± 0.18 b	15.60 ± 0.15 e	19.73 ± 0.23 bcd
Sa-1	14.67 ± 0.25 a	16.67 ± 0.16 c	20.60 ± 0.15 b
Sa-2	13.95 ± 0.19 c	18.35 ± 0.20 a	20.82 ± 0.27 b
Sa-3	13.75 ± 0.43 c	15.00 ± 0.17 ef	21.47 ± 0.32 a
Sa-4	13.22 ± 0.28 cd	16.62 ± 0.18 c	20.28 ± 0.23 bc
Sa-5	13.42 ± 0.24 cd	17.73 ± 0.22 b	20.67 ± 0.26 b
Sa-6	13.88 ± 0.16 c	15.63 ± 0.19 e	18.80 ± 0.20 e
Sa-7	12.98 ± 0.19 e	16.40 ± 0.12 cd	20.02 ± 0.21 bc
Sa-8	12.78 ± 0.27 e	16.87 ± 0.19 c	19.22 ± 0.16 d
F-Value	6.3	35.1	13.0

Sa = *Staphylococcus aureus*
The value of each constituents consisted of ± S.D. of six replicates.
The F value is significantly different when $p<0.05\%$.
In each column the mean value with different alphabetical letters are significantly different

potent activity than that of DCM fraction against almost all strains tested (**Table 4**).

Plasmids are associated with the ability to confer antimicrobial resistance among bacteria. The majorities of the genes mediating resistances to antibiotics are located on plasmid or on transposons and are readily circulated. Plasmid analysis also allows the differentiation of bacterial isolates belonging to the same serotype. Thus, plasmids are an important epidemiological tool for determining the origin of a disease outbreak or for investigating the dissemination of an infectious agent in a population (Clewell 1981; Cantin *et al*. 1992). In view of this in our finding, organisms with different sources recorded different degrees of zones of inhibition depending on the fraction tested (**Tables 2-4**)

According to many reports (Senda *et al*. 1996; Jones *et al*. 2001) multiple resistances in microorganisms are spreading hazardous infectious diseases in the world. An alternative to combat the problem of microbial resistance is to develop new antibacterials for substitution of ineffective ones. In the light of that medicinal plants and microorganisms are the proper candidates for searching novel antimicrobial agents (Shahidi *et al*. 2004). There are reports on the efficacies of pure derivatives of benzopyran against Gram-positive and Gram-negative bacteria as well as fungi (Kayser and Kolodziej 1997; Bisignano *et al*. 2000). And there are also reports on the antibacterial (Gram +ve and –ve) property of berberine alkaloids (Hopp *et al*. 1976; Cowan 1999). A literature review showed that the EA fraction of *P. cerasoides* also contains berberine alkaloids (González *et al*. 1997) and the DCM fraction contains benzopyran derivatives (González *et al*. 1995, 1996; Zafra-Polo *et al*. 1996). In view of this, in our study the EA fraction exhibited potential activity against all the reference and isolated strains of bacteria. Cerasodine and cerasonine are berberine alkaloids which were isolated (González *et al*. 1997) from the EA fraction of the methanol extract of *P. cerasoides*: these two alkaloids may be responsible for the potent antibacterial activity of the same fraction of the methanol extract we extracted from *P. cerasoides*. The DCM fraction showed little antimicrobial activity because the constituents of this fraction, a derivative of benzopyran, may be less effective compared to cerasodine and ceraso-

nine in the EA fraction. Further investigation is required to know the exact constituent responsible for the antimicrobial potency of *P. cerasoides* and its mode of action.

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