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In vitro Efficacy of Flavonoids from *Eugenia jambolana* Seeds Against ESβL-Producing Multidrug-Resistant Enteric Bacteria

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Abstract: Methanol extracts of *Eugenia jambolana* seeds (Myrtaceae), used as a traditional folklore medicine, showed inhibitory effects against the growth of a few multidrug-resistant extended spectrum beta lactamase (ESβL) producing gram-negative bacteria. Bioactivity guided fractionation yielded several fractions. One active flavonoid-containing fraction had a Minimum Inhibitory Concentration (MIC) of 7.9 to 1000 μg mL^{-1}. The strong *in vitro* antibacterial activity of flavonoid derivatives against ESβL producing gram-negative bacteria suggests the compounds might find wide pharmaceutical use.

Key words: Flavonoids, ESβL, minimal inhibitory concentration, thin layer chromatography

INTRODUCTION

Medicinal plants have been used for centuries as remedies for human diseases because they contain components of therapeutic value. Recently, the acceptance of traditional medicine as an alternative form of health care and the development of microbial resistance to the available antibiotics has led authors to investigate the antimicrobial activity of medicinal plants (Bisignano *et al*., 1996; Hammer *et al*., 1999; Lis-Balchin and Deans, 1996). Moreover the increasing antibiotic resistance of several bacteria especially of the extended spectrum β-lactams except cephamycins and carbapenems (Bradford, 2001; Bush *et al*., 1995; Jacoby and Medieros, 1991; Livermore, 1995) is the cause of great concern. Infections due to ESβL-producers often occur in outbreaks and have become a serious problem in hospitalized patients (Bradford *et al*., 1995; Gaillot *et al*., 1998; Kim *et al*., 1998). Moreover, since ESβL-producing organisms are frequently also resistant to aminoglycoside, trimethoprim-sulfamethoxazole and quinolones, the therapeutic choices are limited (Paterson, 2000). Emergence of such resistance raises question about the future of these drugs in chemotherapy, as the transmission of such resistance plasmid to other bacteria will help in the fast dissemination of resistance genes (Hopkins *et al*., 2005). However, β-lactamases continues to be the leading cause of resistance to β-lactam antibiotics in Gram-negative bacteria (Bradford, 2001). Being plasmid mediated, these enzymes spread fast amongst the bacterial population and impacted on chemotherapy. Therefore search for new antimicrobials to combat infectious diseases caused by multidrug-resistant bacteria is urgently needed. Due to poor hygienic conditions in developing countries in both hospital and community, enteric bacterial infection caused by resistant strains are more problematic and of major health problem (Ahmad, 1994; Livermore, 1995). Hence herbal drugs have gained significance now. Though the biological activities of the compounds isolated from *Eugenia jambolana* have been studied (Sridhar *et al*., 2005), yet there are no reports on the effect on the active compound against multi-drug resistant bacteria. However, the selected medicinal plant has not been evaluated for such novel bioactivity.

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MATERIALS AND METHODS

Plant Extract Preparation

The plant used in this study, Eugenia Jambolana Seeds (EJS) were obtained commercially and were identified and authenticated by the Botany department of Holy Cross College, Tiruchirappalli. The seeds were shade dried and powdered. Air-dried powder (1 kg) was extracted with 2 L of respective solvents in a soxhlet apparatus for 18 h. After filtration of the extract, it was evaporated at 30°C until dryness. The obtained crude extract (56 g L^{-1}) was dissolved in n-hexane and then chromatographed on a silica gel column. Initial elution with discontinuous gradient of 50% ethyl acetate and 50% hexane, 75% ethyl acetate and 25% hexane, 100% ethyl acetate and then with a continuous gradient from 90% ethyl acetate and 10% methanol till 100% methanol. This yielded 13 fractions (F1-13). The fractions F_{1-3}, F_{4-6}, F_{7-9}, F_{10} and F_{13} were combined according to their Rf values into five fractions.

Test Organisms

Urinary isolates from symptomatic Urinary tract infected patients attending or admitted to CSI Mission General Hospital in Tiruchirappalli, South India, from October 2005-March 2006, were identified by conventional methods. Nine clinical isolates and three standard strains (Table 1) were included for the study. The bacterial strains were grown and maintained on Nutrient Agar slants.

Screening for ESβL-Production

The antibiogram obtained for the clinical isolates revealed them to be multi-drug resistant isolates. The test isolates were screened for ESβL production following double disc synergy test (Miles and Amyes, 1996). ESβL presence was assayed using the following antiobiotic discs: Cefotaxime (30 μg), cefotaxime/clavulanic acid (30/10 μg), ceftazimide (30 μg) and ceftazidime/clavulanic acid (30/10 μg. *K. pneumoniae* ATCC 700603 and *E. coli* ATCC 25922 served as positive and negative controls, respectively.

Antibacterial Assay

The antibacterial activity of the extract was evaluated by the disc diffusion method (Bauer *et al*., 1996). Mueller Hinton agar plates were prepared and inoculated on the surface with the test organism whose concentration was adjusted using 0.5 STD. McFarland's opacity tube (McFarland, 1907).

Table 1: Antibiogram pattern of the screened ESβL-producers

ESβL-producing organisms (n = 220)	A	Ak	A-C	Cu	G	Nx	Ce	Ca	*I
Escherichia coli (n = 100)	(96)	(21)	(72)	(83)	(73)	(89)	(74)	(78)	S
	96%	21%	72%	83%	73%	89%	74%	78%	
Klebsiella pneumoniae (n = 36)	(34)	(23)	(28)	(26)	(22)	(28)	(29)	(30)	--
	93%	65%	78%	73%	62%	78%	80%	84%	S
Pseudomonas aeruginosa (n = 10)	(10)	(6)	(8)	(10)	(6)	(8)	(9)	(6)	--
	100%	62%	80%	100%	60%	80%	90%	60%	S
Citrobacter freundii (n = 8)	(8)	(4)	(5)	(8)	(4)	(6)	(5)	(6)	--
	100%	50%	60%	100%	50%	80%	60%	70%	S
Acinetobacter banumanii (n = 18)	(18)	(3)	(13)	(9)	(2)	(3)	(3)	(5)	--
	100%	20%	65%	50%	10%	20%	20%	30%	S
Aeromonas hydrophila (n = 20)	(20)	--	(20)	(12)	(20)	(20)	(20)	(20)	--
	100%	S	100%	60%	100%	100%	100%	100%	S
Enterobacter aerogenes (n = 14)	(13)	(4)	(6)	(7)	(4)	(6)	(3)	(5)	--
	90%	30%	40%	50%	30%	45%	20%	35%	S
Proteus mirabilis (n = 4)	(4)	(3)	(3)	(3)	(3)	(3)	(2)	(2)	--
	100%	80%	90%	80%	90%	80%	50%	50%	S
Morganella morganii (n = 10)	(9)	(1)	(2)	(6)	(1)	(2)	(6)	(7)	--
	90%	10%	20%	60%	10%	20%	60%	70%	S

A: Ampicillin (10 mc g); Ak: Amikacin (30 mc g); A-C: Amoxy-clavulanic acid (20/10 mc g); Cu: Cefuroxime (30 mc g); G: Gentamycin (10 mc g); Nx: Norfloxacin (10 mc g); Ce: Cephotaxime (30 mc g); Ca: Ceftazimide (30 mc g); I: Imipenem (10 mc g)

About 10 µL of the test extracts (1 g in 10 mL DMSO) were impregnated on sterile discs (Himedia, Mumbai, India) and on drying; the discs were placed on Mueller Hinton plates. After incubation for 24 h at 37°C, positive results were established by the presence of clear zones of inhibition around the active extracts. Also DMSO and solvent only discs were used as controls. The assessment of the antibacterial activity was based on the measurement of diameter of the zone of inhibition formed around the standard antibiotic discs (NCCLS, 1998).

Determination of Minimal Inhibitory Concentration and Minimal Bactericidal Concentration

Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC) were determined for the extracts and fraction by broth dilution method as described by Ayafor *et al*. (1994). The concentration at which there was no visually detectable bacterial growth was taken as the MIC and the concentration at which there was no bacterial growth after inoculation in Mueller Hinton agar was taken as MBC.

Phytochemical Screening

The most bioactive fraction obtained from the methanol extract of *E. jambolana* was selected for preliminary phytochemical screening. Test for alkaloids, steroids, flavonoids, terpenoids and proteins were carried out according to the methods of Harborne (1973).

RESULTS AND DISCUSSION

The antibiogram of the isolates selected for the study are shown in Table 1. The results of the disc diffusion assay of the alcoholic crude extracts and the fractions of the methanol extracts are listed in Table 2 and 3, respectively. No zones were observed for solvent only discs and Dimethyl Sulphoxide

Table 2: Disc diffusion assay of the crude extracts of EJS (conc. 100 mg mL^{-1})

	Zones of inhibition (diameter in mm)		
Organisms	Acetone	Methanol	Hexane
Acinetobacter baumannii	15.0	20	12.0
Acromonas hydrophila	14.0	14	18.0
Citrobacter freundii	8.2	13.3	7.6
Escherichia coli	6.5	14	5.5
Enterobacter aerogenes	8.0	13	17.0
Klebsiella pneumoniae	18.0	16	8.0
Pseudomonas aeruginosa	13.0	18	14.0
Proteus mirabilis	10.0	15	12.0
**E.coli* ATCC 25922	8.0	18	11.0
***E.coli ATCC 35218*	8.0	12	8.0

* ESβL negative control; ** ESβL positive control

Table 3: Disc diffusion assay of the fractions of the methanol extract of EJS (conc.1 mg mL^{-1})

	Zone of inhibition (diameter in mm)				
Organisms	F_1	F_2	F_3	F_4	F_5
Acinetobacter banumanii	--	13	5	12	16
Aeromonas hydrophila	--	7	--	6	12
Citrobacter freundii	--	--	--	11	20
Escherichia coli	--	--	--	15	17
Enterobacter aerogenes	--	--	--	16	16
Klebsiella pneumoniae	--	--	--	12	19
Pseudomonas aeruginosa	--	--	--	15	20
Proteus mirabilis	--	--	--	--	14
**Escherichia coli ATCC 25922*	7	5	9	14	19
***Escherichia coli ATCC 35218*	--	--	--	7	14

*ESβL negative control; **ESβL positive control

Table 4: MIC values of the fractions of methanolic extract of EJS on ESβL producers

	MIC (μg mL^{-1})				
Organisms	F_1	F_2	F_3	F_4	F_5
Acinetobacter banumanii	1000	31.75	62.5	31.75	15.87
Aeromonas hydrophila	1000	31.75	62.5	31.75	31.75
Citrobacter freundii	500	31.75	62.5	62.50	62.50
Escherichia coli	1000	31.75	62.5	125.00	62.50
Enterobacter aerogenes	1000	62.50	62.5	31.75	31.75
Klebsiella pneumoniae	1000	125.00	125.0	62.50	31.75
Pseudomonas aeruginosa	1000	125.00	125.0	62.50	31.75
Proteus mirabilis	1000	62.50	125.0	62.50	31.75
**Escherichia coli ATCC 25922*	1000	31.75	125.0	62.50	31.75
***Escherichia coli ATCC 35218*	1000	62.50	125.0	62.50	62.50

*ESβL negative control; **ESβL positive control

(DMSO). Among the 5 fractions obtained, F_5 exhibited good antibacterial activity against all the bacterial under study. No activity was found with F_1 and low activity was seen with F_2 and F_3. F_4 was active against only a few ESβL-producing Gram-negative bacteria.

The MIC values of the most active fraction, F_5 ranged between 7.9-125 μg mL^{-1}. The results of the phytochemical screening of F_5 of the methanol extract have shown the presence of flavonoids (Table 4).

β-lactam resistance among clinical isolates is growing problem (Saguinetti *et al*., 2003). Many gram-negative bacilli produce ESβL, which are enzymes that mediate resistance to all β-lactams except cephamycins and carbapenems (Daoud and Hakime, 2003). Compared with ESβL-negative isolates, ESβL-positive isolates are more often resistant to aminoglycosides, ciprofloxacin and cotrimoxazole.

Novel antibacterial actions of plant extracts or phytocompounds have been demonstrated which include inhibition of MDR-efflux pump (Dixon *et al*., 1983) and β-lactamase activity (Tsuchiya *et al*., 1994), anti-antibiotic resistance properties (Batista *et al*., 1994) and R-plasmid elimination (Borris, 1996).

In our study, most ESβL-positive strains resistant to several antibiotics (Table 1) were found to be sensitive to our plant extracts as shown. Fraction 5 exhibited maximum antibacterial activity against all the ESβL isolates under study. Flavonoids, the compound detected in F_5 should be responsible for the antibacterial activity. Flavonoids are known to be synthesized by plants in response to microbial infection (Sakanaka *et al*., 1989). Hence it should not be surprising that they have been found in vitro to be effective antibacterial substances against a wide array of ESβL-producing bacteria. Their activity is probably due to their ability to complex with bacterial cell walls and more lipophilic flavonoids may also disrupt microbial membranes (Sakanaka *et al*., 1992). Catechins, a reduced form of the C_3 unit in flavonoid have been studied to inhibit several bacteria as *Streptococcus mutans*, *Vibrio cholerae*, *Shigella* etc. (Vijayua *et al*., 1995; Pengsuparp *et al*., 1995; Watanbe *et al*., 1996). Reports are also available on the antiviral properties of catechins (Critchfield *et al*., 1996). But our findings, that flavonoids are effective against ESβL-producers may be worth mentioning. Table 4 showing MIC values of 7.9, 15.87 and 62.5 μg mL^{-1} of F_5 against several ESβL producing bacteria tested are remarkable.

During the entire study period, all ESβL-positive isolates were susceptible to flavonoids of F_5, indicating that they can be the potential drugs of choice for treating serious infections caused by ESβL-producing microorganisms. Further characterization of the active compound is under study.

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