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Saponins from *Eugenia jambolana* with Antibacterial Activity Against Beta-Lactamase Producing Methicillin Resistant *Staphylococcus aureus*

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Abstract: The present study was carried out to investigate the role of *Eugenia jambolana* against the beta-lactamase producing *Staphylococcus aureus* and to isolate and identify the putative antibacterial compound based on bioassay-guided fractionation. The test bacteria were resistant to several antibiotics, including several beta-lactams. The crude plant extracts demonstrated zones of inhibition in the range of 18 mm against the chosen test bacteria. On the basis of promising activity, methanol extract was subjected to fractionation, which yielded five fractions. The effective fractions had MIC of 31.75-62.5 $\mu\text{g mL}^{-1}$. Phytochemical analyses and Thin Layer Chromatography (TLC) of the most promising fraction showed the presence of saponins as the active phytoconstituent. The active fraction was further tested for its *in vitro* haemolytic activity to sheep and human erythrocytes and demonstrated no haemolysis at recommended and higher doses. Further studies are needed to elucidate the structure of the compound and establish the mode of action of the compound against these multi drug-resistant bacteria.

Key words: *Eugenia jambolana*, bioactivity, fractionation, saponin

INTRODUCTION

Infectious diseases, the leading cause of premature deaths, in the world is killing almost 50,000 people every day. An increase in antibiotic resistant bacteria is threatening world population with the recurrence of infectious diseases (e.g., Tuberculosis and cholera) that were once thought to be under control at least in developed countries. In the recent years incidence of multi-drug resistance in Gram positive (*Staphylococcus aureus*, *Staphylococcus pneumoniae*), Gram negative (*E. coli*, *Shigella*, *Haemophilus influenzae*) and other bacteria like *Mycobacterium tuberculosis* has been reported from all over the world (Mulligen *et al.*, 1993; Sajduda *et al.*, 1998; Sanches *et al.*, 1998). These multi-drug resistant bacteria have also created additional problems in cancer and AIDS patients.

Methicillin resistant *Staphylococcus aureus* (MRSA) has gained much attention in the last decade, as the MRSA is a major cause of hospital acquired (nosocomial infections) β -lactam antibiotics are the preferred drugs against *S. aureus* infections. Although, *S. aureus* has developed resistance to the β -lactam antibiotics due to the production of chromosomal or plasmid mediated β -lactamases or by producing penicillin binding proteins (PBPs). All the *S. aureus* strains have four PBPs (PBP1 to PBP4), but MRSA express a special PBP (PBP2 or PBP2a) from the *mecA* gene. PBP2a takes over the biosynthetic function of normal PBPs in the presence of inhibitory concentration of β -lactams because PBP2 has a decreased binding affinity to β -lactams (Dennessen *et al.*, 1998; Bachi and Rohrer, 2002). This has resulted in the development of multidrug resistance against β -lactam and other antibiotics. Moreover, increased incidence of vancomycin-resistant MRSA has also been reported (Hiramatsu *et al.*, 1997). Thus, the number of effective exogenous antibiotics is decreasing, therefore

concerted efforts are to be made to identify antimicrobial materials from natural products and traditional medicines. Antimicrobial activity of medicinal plants against drug-resistant bacteria, including MRSA and their interaction with β -lactam antibiotics. Beta-lactamase activity on mec gene product have been reported in one or the other plants, including *Camellia sinensis*, *Rosa canina*, *Scutellaria amoena* and *Arctostaphylos uvaursi*, from different parts of the world (Yam *et al.*, 1998; Shiota *et al.*, 2000; Liu *et al.*, 2000; Shimizu *et al.*, 2001; Palombo and Semple, 2002). Such activities mainly in traditionally used Indian medicinal plants are less explored. India is fortunate in possessing the world's richest flora with about 120 families of plants, comprising of 1,30,000 species. The use of about 2400 of these plants have been mentioned in Ayurvedic and Unani texts and tribals use many others. Various preparations of these medicinal plants are used in treating ailments such as wounds skin diseases, dysentery, cough, cold, jaundice and leprosy etc. (Chaudhary, 1996).

Screening of Indian medicinal plants revealed varying degree of antimicrobial activity against various pathogenic and opportunistic microorganisms (Ahmad *et al.*, 1998; Mehmood *et al.*, 1999; Ahmad and Beg, 2001). It is therefore, expected that such bioactive plant extracts may provide some alternative compounds to be used against MRSA with or without the combination of β -lactam antibiotics. In search of such an activity, crude alcoholic extracts and fractions of some Indian medicinal plants were evaluated for their antibacterial activity against β -lactamase producing-methicillin resistant *S. aureus* isolates and an isolate of methicillin sensitive *S. aureus* (MSSA). Preliminary data on the interaction between active plant extracts and certain antibiotics has also been generated.

MATERIALS AND METHODS

Plant Material

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. Holy Cross College, Trichy, Tamil Nadu, India. The air-dried seeds were powdered and 1 kg was extracted using methanol, acetone and hexane in a soxhlet apparatus and were evaporated to dryness under reduced pressure in rotary evaporator. The yields of the acetone, hexane and methanol extracts were 12.1, 10.9 and 14.3 g %, respectively. The dry residues of the crude extracts obtained were stored for further use. For convenience the methanol, acetone and hexane extracts of *E. jambolana* were named EJM, EJA and EJH, respectively.

Fractionation of the Crude Extract with Promising Results

Fractionation of the crude extract was based on bioactivity. The most bioactive crude extract was chromatographed on a silica gel column. 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 F₁₋₃, F₄₋₆, F₇₋₉, F₁₀ and F₁₃ were combined according to their Rf values into 5 fractions.

Test Organisms

Bacterial isolates were obtained from clinical samples from symptomatic Urinary tract infected patients attending or admitted to CSI Mission General Hospital in Tiruchirappalli, South India, from June 2005-August 2005 (Table 1). The bacterial strains were grown and maintained on Nutrient Agar slants.

Table 1: Determining the antibacterial activity of different extracts of EJS (zones of inhibition in mm)

Strains	Methanol extract	Acetone extract	Hexane extract
MRSA-1	17	13	7
MRSA-2	18	13	7
MRSA-3	18	12	6
MRSA-4	17	13	7
MRSA-5	18	12	6
MRSA-6	18	13	7
MSSA	29	14	9

Screening for ESBL Production

The antibiogram obtained for the isolated bacteria revealed them to be multi-drug resistant clinical isolates. The tested isolates were screened for ESBL production following double disc synergy test (Miles and Amyes, 1996). ESBL presence was assayed using the following antibiotic discs: Cefotaxime (30 µg), Cefotaxime/clavulanic acid (30/10 µg), ceftazidime (30 µg) and ceftazidime/clavulanic acid (30/10 µg.) *E. coli* ATCC 35218 and *E. coli* ATCC 25922 served as positive and negative controls, respectively.

Antibacterial Activity

The antibacterial activity of the extract was evaluated by the disc diffusion method (Bauer *et al.*, 1966). 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). 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, 2002).

Determination of Minimal Inhibitory Concentration of the Plant Extracts

Minimal Inhibitory Concentration (MIC) were determined for the extracts by broth dilution method as described by Ayafor *et al.* (1994), while the MIC of the fractions of the extracts was determined by the microbroth dilution method (Eloff, 1998). 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 Analysis of Plant Extracts

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

Determination of Cellular Toxicity Using Sheep Erythrocytes

The method described by He and Ursula (1994) was employed to study cellular toxicity. Briefly, 10-fold serial dilutions of the extract were made in phosphate buffered saline. A total volume of 0.8 mL for each dilution was placed in an eppendorf tube. A negative control tube (containing saline only) and a positive control tube (containing saponin, 5 mg mL⁻¹) were also included in the analysis. Fresh sheep erythrocytes were added to each tube, to give a final volume of 1 mL. Solutions were incubated at 37°C for 30 min and all tubes were centrifuged for 5 min and then observed for haemolysis. Complete haemolysis was indicated by a clear red solution without any deposit of erythrocytes. Haemolysis was also checked microscopically and presence or absence of intact RBCs.

Table 2: Determination of the minimal inhibitory concentrations of different extracts of EJS (mg mL⁻¹)

Strains	Methanol extract	Acetone extract	Hexane extract
MRSA*	1.6	6.25	6.25
MSSA	0.2	3.20	1.60

*Represents the average of 6 strains in triplicates

Table 3: MIC of the fractions of the methanol extract of *Eugenia jambolana* against MRSA and MSSA clinical isolates

Organisms	MIC values (µg mL ⁻¹)				
	F ₁	F ₂	F ₃	F ₄	F ₅
MRSA	1000	62.50	62.5	31.75	62.5
MSSA	1000	31.75	62.5	31.75	7.9

RESULTS

Antibiotic sensitivity of methicillin resistant *Staphylococcus aureus* (MRSA) strains showed resistance to multi-drugs while a methicillin sensitive *S. aureus* (MSSA) strain was resistant to a few antibiotics tested. The minimum inhibitory concentration (MIC) of β-lactam antibiotics (benzyl penicillin, ampicillin, cloxacillin, cefixime) ranged from 250 to >2000 µg mL⁻¹ against the six strains of methicillin resistant *Staphylococcus aureus* while the MIC values of these antibiotics against the MSSA strain ranged from 1.6 to 25 µg mL⁻¹. Production of β-lactamases was a common characteristic for these MRSA and MSSA strains. The extract of *Eugenia jambolana* (seeds) showed broad-spectrum antibacterial activity against all MRSA and the MSSA strains with inhibition zone size 11-29 mm. The diameters of the zones are even comparable with a few antibiotics. The MIC values of the extracts ranging between 0.2 to 6.2 mg mL⁻¹ are highly significant (Table 2). Phytochemical analysis showed the presence of different phytochemicals like alkaloids, flavonoids, phenols, glycosides, tannins, saponins, steroids and terpenoids. On fractionation, the fractions demonstrated a MIC, ranging between 31.75 and 62.5 µg mL⁻¹ (Table 3). The most potent fraction was found have saponins as the major bioactive phytoconstituent. These extracts demonstrated no haemolytic activity to both sheep and human erythrocytes, confirming their non-toxicity.

DISCUSSION

The choice of drugs, to be used against MRSA, is shrinking day by day as susceptibility of MRSA to drugs is decreasing by target site alteration, enzyme modification and permeability changes (Brumfitt and Hamilton-Miller, 1989). Different substrate specificity/spectrum has been reported in various type of MRSA strains, like β-lactamase positive and PBP2, inducible β-lactamase negative and PBP2 constitutive, chromosomal and plasmid-encoded β-lactamases. Production of β-lactamases may also directly influence the resistance level in these strains since these strains are resistant to methicillin where PBP2 is expected to play major role in the development of resistance against not only with β-lactam antibiotics but also to unrelated antibiotics. The β-lactamase produced by one strain of MRSA also hydrolyzed methicillin. This strain was resistant to β-lactam antibiotics with high MIC values, including methicillin (>100 µg mL⁻¹). Therefore, it is expected that this clinical strain might belong to MRSA group and not to BORSA/MODSA group which confers low level of resistance against methicillin. To confirm the presence of *mecA* gene in this strain, further characterization is needed. In the present study, six multidrug resistant β-lactamase producing MRSA strains and a β-lactamase producing MSSA strain, were used to determine the antibacterial activity to traditionally used Indian medicinal plants. The behavior of *S. aureus* strains, against different extracts differed significantly, which requires further investigations in terms of their active constituents and its effect on the MRSA strains. Our findings are in agreement with the reports on other plants (Yam *et al.*, 1998; Palombo and Semple, 2002). However, this is probably the first report on the chosen Indian medicinal plants against β-lactamase producing MRSA and MSSA strains.

Phytochemical analysis by color test and TLC bioautography of certain extracts revealed the presence of alkaloids, flavonoids, phenols, glycosides, saponins as major phytoconstituents. Phenols and flavonoids showed antibacterial activity against MRSA strain under TLC-bioautography. To identify the exact chemical nature of active compounds in these plant extracts detailed phytochemical investigations are needed. Therefore, activity based fractionations of these extracts and determination of active principle and its mechanism of interaction with antibiotics have to be explored in order to uncover the real therapeutic potential of these extracts in the management of infectious diseases caused by MRSA strains and probably by other multidrug resistant bacteria.

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