

SYNERGISTIC ACTION OF TWO INDIAN MEDICINAL PLANTS ON CLINICAL ISOLATES OF VANCOMYCIN RESISTANT *ENTEROCOCCI* SPECIES

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Summary

Alcoholic extracts of *Eugenia jambolana* (EJ) seeds and *Elephantopus scaber* (ES) whole plant, the two traditionally used Indian medicinal plants were investigated for their ability to inhibit the growth of vancomycin resistant *Enterococci* (VRE). The test bacteria, VRE was resistant to several antibiotics, including vancomycin and carbapenems. Our studies showed synergism between the methanol extract of EJ and the acetone extract of ES against VRE. The two plants in combination demonstrated a zone of inhibition in the range of 4.8 to 20 mm against VRE. Phytochemical analysis of the two crude extracts revealed the presence of flavonoids, terpenoids, alkaloids, steroids and saponins. An evaluation of the R-plasmid elimination or modification from VRE by the plant extracts was confirmed by determining the loss of resistance markers in the cured derivative culture. The plant extracts were further tested for their in vitro haemolytic activity to sheep erythrocytes and demonstrated no haemolysis at recommended doses. Further activity-guided fractionation of active fractions is needed to isolate and characterize the active principle in order to establish the mode of action against the VRE and the mechanism of synergy. These findings suggested that the active compounds of both the plants in combination might be useful in controlling VRE infections.

Keywords: *Eugenia jambolana* ; *Elephantopus scaber*; vancomycin resistance; active compounds.

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Introduction

Bacteria have evolved numerous defenses against antimicrobial agents, and drug-resistant pathogens are on the rise. In the recent years, incidence of multidrug resistance in pathogenic and opportunistic bacteria has been increasingly documented (1). These multidrug-resistant bacteria have also created immense clinical problems in cancer and immune compromised patients. Important multidrug-resistant bacteria on the global scale include Gram-positive vancomycin resistant enterococci (VRE). 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 (2).

Novel antibacterial actions of plant extracts or phytocompounds have been documented which include inhibition of MDR-efflux pump (3), anti-antibiotic resistance properties (4) and R-plasmid elimination (5). Similarly, few plant extracts and phytocompounds exhibited synergistic interaction with each other in combination or with antibiotics against Gram-positive bacteria (6). Screening of crude extracts for synergistic interaction is expected to provide bioactive compounds to be used in

combinational therapy. Such compounds or active fractions may not necessarily have strong antibacterial activity in isolation, but may enhance the activity of when in combination.

Enterococci are constitutive members of the intestinal flora of humans and animals and may also colonize the upper respiratory tract, biliary tracts and vaginas of otherwise healthy persons (7,8,9). *Enterococci* have been documented to cause infection of the urinary tract and other sites (9,10,11,12). Although more than one dozen species of *Enterococcus* have been identified, *Enterococcus faecalis* and *Enterococcus faecium* account for approximately 85–90% and 5–10% of human enterococcal infections, respectively (7,8,9). Urinary tract infections (UTI) are the most common nosocomial infections caused by *Enterococci*, including vancomycin-resistant strains (7,8,9). Urinary tract infections have been reported to account for 34–46% of all infections in the hospital and occur at a rate of 12.9 cases/1000 discharges. In addition to UTI, vancomycin-resistant *Enterococci* have also been associated with asymptomatic bacteriuria, colonization of the urinary tract and symptomatic disease such as cholecystitis, cholangitis, peritonitis, septicemia, endocarditis, meningitis and simple wound infections (9,10,11,12). Management of VRE infections poses a clinical challenge, as these organisms may be resistant to several antimicrobials with unique mechanisms of action (13). There are a considerable number of reports on valuable trials carried out to control the infections caused by VRE (14,15,16). However, further trials would be necessary to discover more reliable methods to adequately control VRE infections. In this context, the use of natural products as anti-VRE agents would be a promising field on the pathway towards the prevention of VRE infections. Further it would be very important to investigate the synergistic behavior of the active natural products, with the hope of enhancing their activity.

Materials and Methods

Plant material

Eugenia jambolana seeds (EJS) were obtained commercially and *Elephantopus scaber* (ES) was collected from Kerala and were identified and authenticated by the Botany department of Holy Cross College, Tiruchirappalli. The voucher specimen is available at the Department of Biotechnology, Holy Cross College, Trichy, Tamil Nadu, India. The air-dried seeds of EJ and ES plants were powdered and 1kg 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 of EJ were 12.1 gm %, 10.9 gm % and 14.3 gm% and of ES were 12.1 gm %, 10.9 gm % and 14.3 gm% 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 and that of *E.scaber* were ESM, ESA and ESH respectively. Further testing were conducted using the more promising EJM and ESA.

Bacterial isolates

About 27 urinary isolates of VRE (*E. faecium*, *E. faecalis* only), collected over a one year period (June 2005-June 2006) from CSI Mission General hospital, Tiruchirappalli, Tamil Nadu, India were included for the study.

Antibiotic susceptibility testing

Before antibiotic susceptibility testing, isolates were cultured twice onto blood agar. All antibiotics for susceptibility testing were obtained as laboratory-grade powders from their respective manufacturers. MICs were determined by standard NCCLS broth microdilution methods with Mueller–Hinton broth and were interpreted using NCCLS breakpoints.

Antibacterial activity

The antibacterial activity of the extract was evaluated by the disc diffusion method (17). 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 (18). About 10 µl of the test extracts (1 gm 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 24h 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 (19).

Determination of minimal inhibitory concentration of the plant extracts.

Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) were determined for the extracts by broth dilution method as described by Ayafor (20) and for the fractions by microbroth dilution method (21). 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.jambolana* was selected for preliminary phytochemical screening. Test for alkaloids, steroids, flavonoids, terpenoids and proteins were carried out according to the standard methods (22).

Curing of plasmid from vancomycin resistant *Enterococci* spp.

The curing of plasmid using ethidium bromide was carried out as described by Singh & Yadava (23) and Ahmad(24). A similar procedure was also adopted when attempting to cure plasmid using alcoholic extracts of the two chosen plants. The minimum inhibitory concentration of the curing agent and plant extracts were determined by broth dilution method against the test bacteria. Nutrient broth tubes containing a range of concentrations were prepared and 0.1 ml of freshly grown culture was inoculated in each test tube. The lowest concentration showing no visible turbidity or no significant viable count compared to control when plated on nutrient agar medium was considered as the MIC of that curing agent/plant extract. A range of sub-MIC concentrations was selected to treat the culture. 0.1 ml freshly grown culture was inoculated in each tube containing different concentrations of curing agents and a control broth tube without curing agent. The tubes were incubated at 37 °C for 18 h. Cell broth was diluted in normal saline and spread on nutrient agar plates and incubated at 37 °C overnight. Isolated colonies were replica-plated onto nutrient agar plates containing antibiotics to which the test bacterium was resistant. A plate without antibiotic was simultaneously also inoculated as control. The experiment was performed in duplicate. Percent curing was determined by taking (the mean count of the colonies from antibiotic agar plates that did not grow/ total mean colonies tested) X100.

Determination of cellular toxicity using sheep erythrocytes

The method described by Xian-guo and Ursula (25) 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) 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.

Results and Discussion

Emergence of multidrug resistance in human pathogenic bacteria has created immense clinical problems in the treatment of infectious diseases. Pharmaceutical companies are now looking for alternative drugs from other sources including animals and plants. Medicinal plants are considered as a potential source of new chemotherapeutic drugs because of their diverse phytochemicals and little or no toxic effect. Antibacterial activity was exhibited by plant extracts irrespective of the drug resistance pattern of the test bacteria and the inhibitory zones were recorded (Table 1). Broad spectrum activity is an apparent indication that the plant extract might have a different mode of action (target site) than commonly used antibiotics. Antimicrobial activity and DNA intercalation properties of the active constituents were reported by other workers (26). The curing ability of plant extract might be due to its active constituent, which may intercalate with the DNA molecule and might have inhibited plasmid replication selectively at sub-MIC concentration. However, additional evidence utilizing an appropriate technique (e.g. electrophoresis or gene probing) is required to confirm and characterize the initial presence and subsequent curing of the R-plasmid.

However, further studies are required using current methods for characterizing resistance elements to assess the effects of the plant extracts upon clinically significant strains of microorganisms. On the other hand, data on *in vivo* toxicity of plant extract, stability of active compounds and protection against infections caused by drug-resistant bacteria are to be generated to determine the therapeutic potential of the two plants. However, in the crude extract synergistic or additive antimicrobial activity due to other phytoconstituents is not ruled out. Our phytochemical analysis revealed the presence of saponins, flavonoids and naphthoquinone in the crude extract (Table 2). Synergistic/additive interaction of plant extracts/ phytochemicals is the basis of several herbal formulations in traditional system of medicine. We have made an attempt to see the possible synergistic interaction between plant extracts for their antibacterial activity. Such interaction studies may provide useful data and clue to further investigate such interacting extract for novel compounds. Such interaction may be helpful in effective herbal formulation, and as well needs further investigation regarding the interacting phytochemicals. Phytochemical analysis of plant extracts indicates that the presence of one or more groups of phytoconstituents like tannins, flavonoids, glycoside, phenols, saponin, etc. is responsible for antibacterial activity alone or in combinations. Further isolation and characterization of antibiotic interacting phytochemicals present in both the plants need to be studied as the active fraction (acetone or methanol) still contains the different groups of active compounds revealed by infrared (IR) study. It is interesting to note that all bioactive extracts could not exhibit *in vitro* toxicity to sheep erythrocytes indicating their non-haemolytic nature. Further studies on isolation of active compounds and their mode of action/target site on ESBL-producing enteric bacteria as well as *in vivo* stability and efficacy are needed to exploit them in the management of infectious diseases caused by such multi drug-resistant bacteria. Elimination of plasmid-mediated drug resistance in pathogenic strains of bacteria is of great practical significance both in chemotherapy of bacterial infection and in microbial genetics. In recent years few reports have appeared about the reversal of antibiotic resistance in bacteria after treatment with certain plant metabolites/extracts. However, reversal mechanisms in all cases are not known.

Table 1: Effect of the crude extracts of the two plants on the growth of vancomycin resistant *Enterococcus* spp.

S.No	Plant extract	Diameter of Zones of inhibition (mm)
1.	<i>Eugenia jambolana</i> methanol extract	5.5
2.	<i>Elephantopus scaber</i> acetone extract	4.8
3.	Both the above extracts synergistically	20

Table 2: Preliminary phytochemical screening of the promising extracts of the two plants

Test	Plant Sample	<i>Eugenia jambolana</i> methanol extract	<i>Elephantopus scaber</i> acetone extract
	Terpenoids		+
Flavonoids		+	+
Steroids		+	+
Glycosides		+	+
Reducing Sugar		+	-
Alkaloid		+	+
Quinones		+	+
Phenols		+	+
Tannins		+	-
Saponins		+	-

Table 3: Plasmid curing effect expressed in terms of MIC (mg/ml)

Curing agent	Vancomycin resistant <i>Enterococcus</i> sp	Vancomycin sensitive <i>Enterococcus</i> sp
Ethidium bromide	1.0	*250.0
EJM	5.0	1.25
ESA	10.0	2.5
EJM+ESA	2.5	0.62

* μ G/ml

EJM- *Eugenia jambolana* methanol extract

ESA- *Elephantopus scaber* acetone extract

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