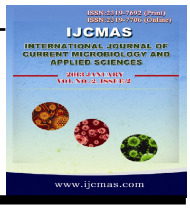
International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7692 **Volume 2 Number 2 (2013) pp.6–14**
http://www.ijcmas.com



Original Research Article

Antibacterial activity of three medicinal plants against clinically isolated multidrug resistant *Enterococcus faecalis* (MDRE)

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Keywords

Antibacterial activity;
Medicinal plants;
multidrug resistant;
Enterococcus faecalis;
MDRE

A B S T R A C T

A total of 100 clinical samples were collected from different hospitals and microbiology laboratories in Kancheepuram district, Tamilnadu, India that includes Blood (15 samples), Pus swabs (25 samples), Stool (40), Urine (20 samples) for screening the *E. faecalis*. Among the 100 clinical samples 47 isolates showed Enterococcus faecalis. The bacterial isolates from the clinical specimens for the present study were confirmed as *E. faecalis* by using conventional tests. The antibiotic resistant of *E. faecalis* isolates showed a maximum of 53.1 percent to Penicillin-G which was followed by other antibiotics in the following order: kanamycin>Streptomycin> Ciprofloxacin>chloramphenical>amphicillin>Vancomycin. The leaves of three plant species *Aegles maromoles, Aristolochia indica and Ocimum canum* were collected and subjected to ethanolic extraction. The ethanolic leaves extracts were tested against clinically isolated Multidrug resistance Enterococcus faecalis and Standard strains of *E. faecalis*. Antibacterial reference standard, Vancomycin had equal effect on Multidrug resistant *E. faecalis* isolates from clinical isolates and Standard *E. faecalis.* Zone of inhibition of Vancomycin was compared with plant extracts. The antimicrobial activity of plant extract was higher in Hibiscus Sabdariffa than the other leaves extract tested against test bacteria. Zone of inhibition of the plant leaves extracts from Hibiscus Sabdariffa showed high antimicrobial activity against Multidrug resistant *E. faecalis*. There is a scope to use ethanolic extract of the leaves of *Aristolochia indica* against Enterococcal infections caused by Multidrug resistance *E. faecalis*.

Introduction

Enterococci are common inhabitants of intestinal tracts of humans and animal are consider as important causes of hospital acquired infection. They are the second most common cause of nosocomial infections and the third most common cause of nosocomial bacteremia (Ike, *et al.*, 1987). Within recent years, a great deal has been learned about the epidemiology and risk factors for nosocomial enterococcal infection, with well documented person-to-person spread of antibiotic-resistant isolates. Enterococci are also important causes of community-acquired infection. These enterococcal

infections have been traditionally thought of as endogenous and arising from the patient's own flora. Microorganisms from endogenous sources subsequently cause infection by invasion of commensal flora which infect because of some alteration in host defenses. The sources and reservoirs that play a role in the resistance to antibiotics of enterococci that are community acquired are not known. In earlier studies in all over the world, community acquisitions of gentamicin-resistant enterococci and of glycopeptide-resistant enterococci (GRE), respectively, were documented, suggesting a reservoir in the community.

The search for plants with antimicrobial activity has gained increasing importance in recent years, due to a growing worldwide concern about the alarming increase in the rate of infection by antibiotic-resistant microorganisms. Numerous studies have been conducted with the extracts of various plants, screening antimicrobial activity as well as for the discovery of new antimicrobial compounds (Cowan, 1999; Raskin *et al.,* 2002). In many developing countries, about 80% of available therapeutic substances are obtained from medicinal plants. Since the most infectious diseases are of a microbiological origin, with the advent of ever-increasing resistant bacterial strains, there has been a corresponding rise in the universal demand for natural antimicrobial therapeutics.

Enterococci have been known to be resistant to most antibiotics used in clinical practice. Multidrug-resistant and Vancomycin-resistant enterococci are commonly isolated from humans (Mark *et al.,* 1991), animal sources, aquatic habitats, foods (Giorgio Giraffa, 2002) and agricultural run-off (Rice *et al.,* 1995), which indicates their ability to enter the human food chain. The purpose of this study was to characterize the antibiotic resistance profiles of *Enterococcus* species isolated from clinical samples.

For a long period of time, plants have been a valuable source of natural products for maintaining human health, especially in the last decade, with more intensive studies for natural therapies. The use of plant compounds for pharmaceutical purposes has gradually increased world wide. About 80% of individuals from developed countries use traditional medicine, which has compounds derived from medicinal plants. Therefore, such plants should be investigated to better understand their properties, safety and efficiency.

Hence, more studies pertaining to the use of plants as therapeutic agents should be emphasized, especially those related to the control of antibiotic resistant microbes. The objective of this research was to evaluate the potential of plant extracts and phytochemicals on standard microorganism strains as well as multi-drug resistant bacteria, which were isolated from clinical samples.

Materials and Methods

Sample collection:

Samples for the study were collected form the patients of different Hospital and Microbiology laboratories at Kancheepuram, TamilNadu, India. Clinical specimens such as burn blood (15 samples), pus swabs (surgical and non-surgical wounds) (25 samples), stool (40 samples), and urine (20 samples) specimens of patient, were collected aseptically by using sterile cotton swabs. Stool and urine specimens were collected by using sterile container. The surface area of wound was sterilized by alcohol soaked cotton, the pus samples were collected with a sterile swab and were kept

in Bile esculin medium in order to maintain the viability of samples. After collecting urine samples in sterile containers, 1.5% of boric acid was added for controlling the overgrowth. Each sample was labeled with the needed particulars such as name, age and chemotherapy if under taken and the sample was brought to laboratory with in 6 hrs.

Bacterial isolation and conventional phenotypic characterization of Enterococci

The clinical specimens were plated on 5% sheep blood agar. The specimens were also plated on blood agar and Macconkey agar for the isolation of concomitant organisms along with enterococci. Enterococci were identified on the basis of growth on bile-esculin medium, gram staining i.e. gram positive cocci in pairs and short chains, catalase-negative or pseudocatalase positive, growth in 6.5% NaCl and at pH 9.6. Extensive phenotypic and physiological characterization was carried out by the conventional tests devised by Facklam and Collins (1989).

Identification of species

Enterococcal strains were further identified up to species level by using conventional physiological tests devised by Facklam and Collins (1989) which are based on carbohydrate fermentation using 1% solution of the following sugars: glucose, mannitol, rabinose, raffinose, sorbitol, sucrose, lactose, trehalose and inulin; by pyruvate utilization in 1% pyruvate broth; arginine decarboxylation in Moellers decarboxylase broth; hippurate hydroloysis; motility test; detection of pigment production on tryptic soy agar (TSA); gelatin liquefaction; starch hydrolysis using 2% starch and polysaccharide production. Ability to produce enzyme phosphatase by *Enterococcus* species was also tested using phenolphthalein-phosphate agar, haemolysin production was detected in the strains of *E. faecalis* by culturing the isolates on blood agar using 5% human blood. A single colony isolate was inoculated into 5mL Todd-Hewitt broth and incubated overnight at 37° C which was then added as an inoculum of one drop with the help of Pasteur pipette (Albert Manero and Blanch, 1999). All tests were incubated at 37° C and read at 24 hours and 7 days.

Antibiotic sensitivity test:

Antibiotic susceptibility testing of the clinical isolates along with the quality control strains were performed using Brain Heart Infusion agar instead of Muller Hinton agar by disk diffusion method. Antibiotic susceptibility test was conducted by adopting Kirby-Bauer disc diffusion method. The cultures were streaked closely with swab on the medium in the form of lawn. In the plate antibiotics such as Penicillin-G (10 µg/disc), ampicillin (10 µg/disc), kanamycin (30 µg/disc), streptomycin (30µg/disc), ciprofloxacin (5µg/disc), vancomycin (30µg) and chloramphenical (30 µg/disc) discs were placed and incubated at 37°C. Following overnight incubation the plates were examined for areas of no growth around the disc (zone of inhibition) (Bauer *et al.*, 1966).

Results

Morphology of adult female and male

Among the 100 clinical samples 47 isolates showed positive for *Enterococcus faecalis* (Table 2). The highest isolates were obtained in stool samples. The results on percentage of each samples showed positive for *Enterococcus faecalis* isolates was tabulated in Table-3.

Table.1 Phenotypic characteristics of *Enterococcus faecalis* isolated from clinical samples (n=100)

Sl. No.	Phenotypic characteristic test	Activity/Observation
1.	Gram Reaction	Gram positive cocci in clusters
2.	Motility	Non motile
3.	Growth on nutrient agar	Yellow colonies
4.	Growth on blood agar	β - hemolysis
5.	Growth on KF Agar	Red colour colonies
6.	Growth on Bile Esculin azide agar	Black colour formation
7.	Growth on Esculin agar	Black colour formation
8.	IMViC	
	(i) Indole	Negative
	(ii) Methyl red	Negative
	(iii) Voges-Proskauer	Positive
	(iv) Citrate	Positive
9.	Gelatin liquefaction	Positive
10.	Catalase	Negative
11.	Oxidase	Positive
12.	Urease	Positive
13.	Ariginine dihydrolase	Positive
14.	Growth on pH-9.6	Growth occurs
15.	Growth on Nacl-6.5%	Growth occurs
16.	Phosphatase production (Phenolphthalein phosphatase agar)	Positive
17.	Growth on Starch medium	Blue colour zone formation around the colonies
18.	Growth on Trypticase medium	Yellow colour colonies due to pigment production
19.	PYRase test	Positive

Table.2. Total number of *Enterococcus faecalis* isolated from clinical samples

S.No.	Clinical samples (n=100)	No. of *Enterococcus faecalis* positive	Percentage of *Enterococcus faecalis*
1	Blood (15 samples)	7	46.6
2.	Pus swabs (25 samples)	11	43.4
3.	Stool (40 samples)	21	60.00
4.	Urine (20 samples)	8	40.00

Table.3 Antibiotic resistance percentage of *Enterococcus faecalis* isolates from clinical samples (n=100)

S. No.	Nature of sample(total no. of samples collected)	No. of *Enterococcus faecalis* isolates used.	Antibiotics used (% of isolates showing reisitance)						
			PG	CP	KM	SM	CF	AC	VA
1.	Blood (15)	7	57.1 (4)	28.5 (2)	42.8 (3)	42.8 (3)	57.1 (4)	28.5 (2)	42.8 (3)
2.	Pus swabs (25)	11	45.4 (5)	36.3 (4)	45.4 (5)	54.5 6)	36.3 (4)	27.2 (3)	27.2 (3)
3.	Stool (40)	21	57.1 (11)	42.8 (9)	57.1 (11)	47.6 (10)	33.3 (7)	28.5 (6)	23.8 (5)
4.	Urine (20)	8	62.5 (5)	12.5 (1)	50.0 (4)	37.5 (3)	25.0 (2)	37.5 3)	25.0 (2)

The number of resistant isolates in parentheses ( ).

PG- Penicillin-G; CP-Chloramphenicol; KM-Kanamycin; SM-Streptomycin; CF-Ciprofloxacin; AC-Ampicillin; VA-Vancomycin.

Table.4 Effect of ethanolic extracts of selected plant leaves on Multidrug resistance *Enterococcus faecalis*

S.No.	Ethanolic extract of the plant leaves used	Zone of inhibition in cm	
		IMREF	SEF
1	*Aegles maromoles*	1.8	1.8
2	*Aristolochia indica*	1.9	2.0
3	*Ocimum canum*	1.4	1.5

Table.5 Antibacterial reference standards against Multidrug resistance *Enterococcus faecalis*

Antibiotic	Concentration (µg/disc)	Test Bacteria	Zone of inhibition (cm)
Vancomycin	30 µg	IMREF SEF	2.1 2.2

(Values are mean of three replicates)

IMREF – Clinically isolated Multidrug resistance *Enterococcus faecalis;*
SEF – Standard *Enterococcus faecalis;*

The phenotypic characteristics of *Enterococcus faecalis* isolates from clinical samples are indicated in Tables 1. Preliminary identification of the isolates was performed by conventional tests (gram-positive cocci, catalase negative, growth at 45°C, growth in 6.5% Na Cl, and bile-esculin positive). Additional biochemical tests were performed as previously described to confirm speciation, including production of acid in the presence of lactose, mannitol, fructose, glucose, mannose and sucrose.

Out of 100 samples, 47 samples showed the presence of *Enterococcus faecalis*. The antibiotic resistant of *Enterococcus faecalis* isolates showed a maximum of 53.1 percent to Penicillin-G which was followed by other antibiotics in the following order: kanamycin> streptomycin> ciprofloxacin> chloramphenical>amphicillin>vancomycin (Table 3).

The ethanolic extracts of the plant leaves includes *Aegles maromoles, Aristolochia indica* and *Ocimum canum*showed antimicrobial activity against multidrug resistant *Enterococcus faecalis* (Table.4). Antibacterial reference standard, vancomycin had equal effect on multidrug resistant *Enterococcus faecalis* tested and also tested for standard *Enterococcus faecalis* strains (Table 5).

The result on the analysis of ethanolic extracts of the plant leaves includes *Aegles maromoles, Aristolochia indica* and *Ocimum canum* showed antimicrobial activity against Multidrug resistant *Enterococcus faecalis* and Standard *Enterococcus faecalis* that include 1.9 and 2.0 respectively (Table 4). Among the plant leaves tested *Aristolochia indica* showed strong activity against Multidrug resistant *Enterococcus faecalis*. Ethanolic extracts of *Aegles maromoles* showed moderate antimicrobial activity against Multidrug resistant *Enterococcus faecalis* and Standard *Enterococcus faecalis* strains (Table 4). Antibacterial reference standard, vancomycin had equal effect on Multidrug resistant *Enterococcus faecalis* tested and also tested for standard *Enterococcus faecalis* strains (Table.5).

Discussion

Enterococci are not generally regarded as highly virulent bacterial pathogens; however, resistance to many antimicrobial drugs complicates the treatment of enterococcal infections. Acquired resistance to high concentrations of ampicillin, aminoglycoside, and glycopeptide antibiotics, specifically vancomycin, has exacerbated this problem.

The results showed the prevalence of *Enterococcus faecalis* from 100 various clinical samples. About 46% (7 isolates from blood, 43.4% (11 isolates) from pus, 60% (21 isolates) of stool and 40% (8 isolates) of urine sample, *Enterococcus faecalis* have been isolated and characterized. This study is undertaken to reveal the emergence of multidrug resistance of *Enterococcus faecalis* which posing a serious therapeutic challenge. *Enterococcus faecalis* was found to be most predominant in stool samples rather than in other samples and least percentage of isolates have been observed in urine samples.

The parallel reports have been reported by Desai, *et al.,* (2001) from hospitalized patients. A total of 236 *Enterococcus faecalis* were identified and their susceptibilities to 11 antibiotics were also determined. Overall 195 (82.6%) isolates

were *Enterococcus faecalis* shown high level ampicillin and gentamicin resistant.

Out of 100 samples, 47 samples showed *Enterococcus faecalis*. All isolates of *Enterococcus faecalis* subjected to antimicrobial sensitivity test. The highest isolates (21) were obtained in stool samples. It reveals the emergence of multi-drug resistance among unusual species of enterococci posing a serious therapeutic challenge. Precise identification of enterococci to species level enables us to access the species-specific antimicrobial resistance characteristics, apart from knowing the epidemiological pattern and their clinical significance in human infections.

The nature of bacterial isolates was identified to include in species level using standard morphological, biochemical, and cultural characteristics (Table 1), with the help of Facklam and Collins (1989), the bacterial isolates from the clinical specimens for the present study were confirmed as *Enterococcus faecalis.*

This study was carried with an aim to isolate Multidrug resistant *Enterococcus faecalis* from Clinical samples from different medical laboratories and subjected to antibiotic sensitivity test by using Penicillin-G, Chloramphenical, Kanamycin, Streptomycin, Ciprofloxacin, Ampicillin, Vancomycin. But, the Vancomycin showed highly sensitive to *Enterococcus faecalis.* The antibiotic resistant of *Enterococcus faecalis* isolates showed a maximum of 53.1 percent to Penicillin-G which was followed by other antibiotics in the following order: kanamycin>Streptomycin>Ciprofloxacin> chloramphenical>amphicillin>Vancomycin (Table 3).

Enterococci are not generally regarded as highly virulent, however resistance to many antimicrobial drugs complicates in the treatment of *Enterococcal* infections. Treatment of Multidrug resistant Enterococci is under an investigation for new drug program for the treatment of patients with life threatening infection especially vancomycin resistant *Enterococcus faecalis* bacteremia. Linezolid is one of the investigational agents but clinical efficacy and safety studies are still not determined.

Hence, the present study clearly indicates an effect to develop an alternative medicine against multidrug resistant *Enterococcus faecalis*, an in vitro susceptibility test of the leaves extracts of three species of plants, was used.

The results on the analysis of ethanolic extracts of the plant leaves includes *Aegles maromoles*, *Aristolochia indica and Ocimum canum* showed antimicrobial activity against Multidrug resistant *Enterococcus faecalis* and standard *Enterococcus faecalis* that include 1.9 and 2.0 respectively (Table.4). Among the plant leaves tested *Aristolochia indica* showed strong activity against Multidrug resistant *Enterococcus faecalis*. Ethanolic extracts of *Aegles maromoles* showed moderate antimicrobial activity against Multidrug resistant *Enterococcus faecalis* and Standard *Enterococcus faecalis* strains (Table.4). Antibacterial reference standard, vancomycin had equal effect on Multidrug resistant *Enterococcus faecalis* tested and also tested for standard *Enterococcus faecalis* strains (Table.5).

Ali *et al*., (2005) reported phytochemical, pharmacological and toxicological properties of *Aristolochia indica* showed antimicrobial activity against Enterococcus *faecalis*. It exhibits effective antimicrobial activity against strain of *E. faecalis.* The plant species include

Aristolochia indica showed highest antimicrobial activity that 1.9 and 2.0 cm against clinically isolated *Enterococcus faecalis* and Standard strain of *Enterococcus faecalis* respectively, in this present study.

The parallel results have also been reported by Mounnissamy *et al.,* (2002). Gossypetin (3,5,7,8,3,' 4'-hexahydroxy flavone) isolated from the leaves of *Aristolochia indica* has been tested for its antibacterial activity. The antibacterial activity was compared with chloramphenicol against *Escherichia coli, Staphylococcus aureus, Bacillus subtilis, Bacillus pumpilus, Enterococcus faecalis* and *Pseudomonas aeruginosa*. The drug possesses excellent antimicrobial activity against all the test organisms. This activity may be due to polyphenolic nature of the flavonoid Gossypetin.

Since the development of resistance to antibiotics by the pathogenic strains of *Enterococcus faecalis* is an ever increasing problem, a suitable and possible alternate chemotherapeutic compounds which are of plant origin i.e., phytochemical compounds such as alkaloids, terpenoids, polyphenols, flavonoids and steroids are tried for effective means of controlling Multidrug resistant bacteria like *Enterococcus faecalis.*

The present study provides evidence that can be used in subsequent risk assessment exercises to elucidate the role of clinical sample in the dissemination of antibiotic resistance *Enterococcus faecalis* to human populations. This has lead to the emergence and dissemination of resistant bacteria and resistance genes in humans. The main sector of resistance-increasing medicine usage, in regard to human health, lies within the health care sector. Monitoring of antibiotic resistance, antibiotic resistance transfer, and antibiotic use and studies on the dissemination of antibiotic resistance in humans is essential to obtaining consistent and reliable data on the epidemiology of resistance of Enterococcal isolates from humans for the treatment of Enterococcal diseases.

However, data such as those presented here offer evidence that should be helpful in the identification of future study topics and initiatives aimed at reducing the public health burden of antibiotic-resistant pathogens.

Plant extracts have great potential as antimicrobial compounds against microorganisms. Thus, they can be used in the treatment of infectious diseases caused by resistant microbes. There is a possibility of using plant extracts against Multidrug resistance *Enterococcus faecalis* has been observed from the results. There is a scope to use ethanolic extracts of leaves, *Aristolochia indica* against Multidrug resistance *Enterococcus faecalis.*

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