In vitro antibacterial activity of seven Indian spices against high level gentamicin resistant strains of enterococci

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Abstract

Introduction: The aim of the study was to explore the *in vitro* antibacterial activity of seven ethanolic extracts of spices against high level gentamicin resistant (HLGR) enterococci isolated from human clinical samples.
Material and methods: Two hundred and fifteen enterococcal strains were isolated from clinical samples. High level gentamicin resistance in ethanolic extracts of cumin (*Cuminum cyminum*), cinnamon (*Cinnamomum zeylanicum*), ginger (*Zingiber officinale*), fenugreek (*Trigonella foenum-graecum*), cloves (*Syzygium aromaticum*), cardamom (*Elettaria cardamomum* Maton) and black pepper (*Piper nigrum*) were prepared using Soxhlet apparatus. The antibacterial effect of the extracts was studied using the well diffusion method. Statistical analysis was carried out by χ^2 test using SPSS 17 software.
Results: Only cinnamon and ginger were found to have activity against all the isolates, whereas cumin and cloves had a variable effect on the strains. Fenugreek, black pepper and cardamom did not show any effect on the isolates. The zone diameter of inhibition obtained for cinnamon, ginger, cloves and cumin was in the range 31–34 mm, 27–30 mm, 25–26 mm and 19–20 mm respectively.
Conclusions: *Cinnamomum zeylanicum* and *Z. officinale* showed the maximum antibacterial activity against the enterococcal isolates followed by *S. aromaticum* and *C. cyminum*. The findings of the study show that spices used in the study can contribute to the development of potential antimicrobial agents for inclusion in the anti-enterococcal treatment regimen.

Key words: kitchen spices, Soxhlet apparatus, antibiotic sensitivity testing.

Introduction

Enterococci have emerged as an important nosocomial pathogen in the last decade. They can cause a wide variety of infections including urinary tract infections, bacteremia, endocarditis, meningitis and soft tissue infections. Of 47 species known to date, *Enterococcus faecalis* accounts for approximately 80–90% of the clinical isolates and *E. faecium* was found in the remaining cases [1, 2].

The recent interest in *Enterococcus* is due to the increased incidence of nosocomial infections caused by it throughout the world. It stands second in causing nosocomial infections [3]. Resistance of these organisms to the current and the commonly used antibiotics is

one of the burning issues globally. The strains of enterococci have intrinsic as well as acquired resistance to most antimicrobial agents [3].

A common regimen for treatment of serious enterococcal infections such as septicemia is the combination of cell wall inhibitors such as penicillin, ampicillin or vancomycin with aminoglycosides such as streptomycin or gentamicin [4]. The addition of a cell wall inhibitor agent helps in the penetration of the aminoglycoside into the bacterial cytoplasm, making the intrinsically resistant organism aminoglycoside sensitive. The presence of high level aminoglycoside resistance (HLAR) in enterococci defined as the minimum inhibitory concentration of aminoglycoside for the isolate makes the synergism of cell wall inhibitor and aminoglycoside ineffective [5].

In recent years, enterococcal isolates demonstrating high level resistance to both streptomycin and gentamicin, conditions that preclude synergistic interactions between cell wall-active antibiotics and available aminoglycosides, have become common in many health settings [5, 6]. Against such isolates, there is no other combination of antimicrobial agents which reliably provides bactericidal activity.

The possession of diverse virulence factors has been an important benefit for enterococci, since possessing any of them may change the severity of infections caused by these bacteria. On the other hand, it is believed that nosocomial enterococci might have virulence elements that increase their ability to colonize hospitalized patients [4, 8, 9]. Resolution of the high level gentamicin resistant enterococci infections is further complicated by the presence of virulence factors present in these organisms [9].

The shift in the susceptibility of enterococci to antibiotics is a major challenge faced by the medical fraternity and has made the future management of the infections uncertain. Hence it has become the "need of the hour" to find a solution for the growing problem of drug resistance. The increasing failure of antibiotics has led to the screening of several plant extracts for potential antimicrobial activity. If found effective, such alternative antimicrobial agents could be explored further to overcome the problem of microbial drug resistance to antibiotics. To the best of our knowledge no studies have been performed to detect the antimicrobial effect of Indian spices on multi-drug resistant strains of enterococci possessing virulent traits. All the related studies have only dealt with standard strains of enterococci but not with the clinical isolates.

Material and methods

Over a period of 2 years 10,398 clinical samples from patients (120 males and 95 females) attending the tertiary care center were processed in the microbiology laboratory, out of which 215 strains of enterococci were isolated. The samples including blood, urine, catheter tube tip, sputum, vaginal swabs, body fluids, tip of endotracheal tube, and pus were processed to isolate the above-mentioned enterococcal strains. These isolated strains were considered clinically significant when obtained in pure culture from the clinical samples or in significant numbers as part of mixed cultures. The isolates were further identified in accordance with the standard procedure [9, 10].

Presumptive identification

Samples were cultured on to 5% blood agar and MacConkey agar. Gram positive cocci showing negative catalase reaction, positive PYR test, black colonies on bile esculin agar and growth in 6.5% NaCl broth were presumptively identified as enterococci. Further, growth of the isolates at both 4°C and 45°C confirmed them to be enterococci. The bacitracin sensitivity test was also done to exclude other *Streptococcus* species.

Characterization and speciation of isolates

The isolates which were primarily identified as *Enterococcus* were then further characterized to the species level with the help of conventional biochemical methods as devised by Facklam and Collins [10]. This was based on fermentation of the carbohydrates by using 1% solution of the sugars glucose, lactose, raffinose, arabinose, sorbose, sucrose and sorbitol, pyruvate utilization using 1% pyruvate slant, arginine decarboxylation using Moeller's decarboxylation broth motility test and pigment production using nutrient agar.

Screening of isolates for gentamicin resistance

The isolates were tested for high level gentamicin resistance by the Kirby-Bauer disc diffusion method. A high level gentamicin disc (120 µg) was used for the method. The inoculum was prepared according to the McFarland standard 0.5.

Minimum inhibitory concentration (MIC) testing for gentamicin

The MIC testing was performed by using Epsilometer test strips for gentamicin which had a range of 0.064–1064 µg/ml.

Spices

Seven types of typical Indian spices and herbs, namely *Piper nigrum* (black pepper), *Cuminum*

cyminum (cumin), *Trigonella foenum-graecum* (fenugreek), *Cinnamomum zeylanicum* (cinnamon), *Elettaria cardamomum* Maton (cardamom), *Syzygium aromaticum* (cloves) and *Zingiber officinale* (ginger) in the form of whole fruit, seeds, rhizome, bark, and buds were purchased from a local retail market. All the spices were washed thoroughly with distilled water to make them free from any contaminated particulate matter. The spices were then air dried and used for further procedures.

Extraction of spices

The extraction of spices was carried out according to a procedure described by Manoj Kumar Singh *et al.*, which is as follows [11].

Preparation of powdered spices

The air dried materials were powdered using a mixer grinder.

Soxhlet extraction

One hundred and fifty g of the powdered plant material was subjected to Soxhlet extraction using ethanol (500 ml) as an extracting solvent. The extraction temperature was 78°C. The time of extraction was 3–6 h or until the color solvent appeared in the siphon. The crude extract was kept at room temperature for ten days to remove any solvent remaining in the extract. The extracts were stored in dark colored bottles, labeled and stored at 4°C until use.

Preparation of working solution

The crude extract was dissolve in dimethyl sulfoxide (DMSO) (10–40%) by dissolving 1 g extract in 10 ml of it. The concentration of the stock solution was 100 mg/ml. The solutions were stored in a refrigerator.

Inoculum preparation

Three or four colonies of bacteria were transferred to a test tube containing 5 ml of sterile nutrient broth. It was incubated at 37°C for 3 or 4 h. The tubes were compared with McFarland Nephelometer Standard 0.5 (turbidity standard). A blank nutrient broth was used as a control.

Evaluation of antibacterial activity of spice extracts

The crude extracts of spices were screened for their antimicrobial activity against the organisms by the agar well diffusion method presented by Dingle *et al.* [12]. A sterile cotton swab was dipped into the prepared inocula and seeded all over the Mueller Hinton agar plate by rotating through an angle of 60°. After each swabbing, finally the swab was passed round the edges of the agar surface and left to dry for a few minutes at room temperature with the lid closed. Then with the help of a sterile cork borer (6 mm), wells were made in the inoculated plate and labeled. Fifty µl of the working suspensions of the spice extracts were dispensed in the respective wells with the help of the micropipette. The solvent DMSO itself was tested for its activity as a control at the same time. The plates were left for half an hour with the lid closed. Then the plates were incubated at 37°C for 24 h. The antibacterial activity was evaluated by measuring the zone of inhibition in millimeters. Tests were carried out in triplicate.

Results

A total of 215 enterococcal strains were isolated from different clinical samples. Ninety-one (42%) strains of enterococci were isolated from urine samples, 47 (22%) from pus, 21 (10%) from sputum, 18 (8%) each from blood and catheter tip and 9 (4%), 6 (3%), 3 (1%) and 2 (1%) from endotracheal tip, pleural fluid, cerebrospinal fluid, and vaginal swabs respectively.

From 215 strains of enterococci, 148 (69%) strains showed high level gentamicin resistance. Among 148 HLGR, 102 (69%) and 42 (28%) were *E. faecalis* and *E. faecium* respectively and 4 (3%) were unusual enterococcal species (Table I).

The result of the disc diffusion method (120 µg) was in full concurrence with that of the E test method (0.016–1064 µg) in the case of gentamicin. All the 148 (69%) strains which showed HLGR with the HLG disc were resistant at different levels when tested with E-strips. Fifty-six (38%) of the 148 HLGR enterococci had a MIC of > 1024 µg/ml (Tables II, III).

The antimicrobial activity of the seven spices was tested against the multidrug *Enterococ-*

Table I. Distribution of high level gentamicin resistance among different species of enterococci using the Kirby-Bauer disc diffusion method

E strip	*E. faecalis* (*N* = 113) *n* (%)	*E. faecium* (*N* = 70) *n* (%)	Non-faecalis and non-faecium (*N* = 32) *n* (%)				
HLG	102 (90)	42 (60)	*E. avium* (*n* = 11)	*E. casseliflavus* (*n* = 5)	*E. durans* (*n* = 6)	*E. gallinarum* (*n* = 21)	*E. raffinosus* (*n* = 8)
			2 (18)	0	1 (16)	0	1 (25)

Table II. High level gentamicin susceptibility pattern of enterococci isolates by Epsilometer test

Organism	Sensitivity pattern (CLSI)		
Enterococci species	**Sensitive**	**Intermediate**	**Resistant**
	< 500 µg/ml	–	> 500 µg/ml
	67 (31%)	0	148 (69%)

Table III. Distribution of MIC ranges of HLG for various enterococcal species

Enterococci	128	192	256	384	512	768	1024
Number of isolates at specified MIC [µg/ml]	NA	NA	NA	NA	55	37	56

cus by using the agar well diffusion assay. The comparative bioactivity of the spices is shown in Figure 1.

The crude ethanolic extract of cinnamon, cloves, ginger, cardamom and cumin showed significant antibacterial activity against all the clinical isolates of enterococci. The spices differed in their antimicrobial effect, as shown in the Figure 2.

The maximum antibacterial effect was shown by cinnamon and ginger with all 215 isolates inhibited when tested with it. The maximum diameter of the zone of inhibition against the isolates was achieved by cinnamon (34 mm), followed by cloves (26 mm), ginger (20 mm), cardamom (18 mm), and the least was shown by cumin (14 mm). Fenugreek and black pepper did not have any antibacterial effect on enterococcal isolates.

Tables IV and V show that all the different species of *Enterococcus* were maximally inhibited by cinnamon and ginger, followed by cloves. Cumin had an inhibitory effect only on one isolate of *E. faecalis* and *E. faecium* each, whereas cardamom showed antibacterial activity against seven isolates of *E. faecalis* and one *E. faecium* isolate.

The χ^2 test was employed to compare the antibacterial activities of all the spices. Significant differences in activity were found between cinnamon and cloves (p = 0.03), as well as between cinnamon and cardamom (p = 0.020). Similarly, a difference in the bioactivity was found for ginger and cloves as well as ginger and cardamom.

Discussion

It is becoming more and more common to find many bacteria developing resistance to most common antibiotics. Enterococci exhibiting HLGR have been reported as an important cause of hospital acquired infection from many geographical locations, including European countries and the United States [13, 14]. Gentamicin together with a cell wall active agent is one of the common antibiotics used against serious enterococcal infections [4]. HLGR among enterococci poses a serious therapeutic challenge in such serious diseases [15].

The present study investigated the status of high level gentamicin resistance among the different species of enterococci and studied the bioactivity of seven Indian spices on the HLGR strains. Sixty-nine perect of the 215 enterococcal isolates were resistant to high level gentamicin. The results of the disc diffusion method and E test were in absolute concurrence. It was also seen that 3% of the unusual species of *Enterococcus* were also HLGR. We had a clear dominance of HLGR *E. faecalis* (90%) followed by HLGR *E. faecium* (60%).

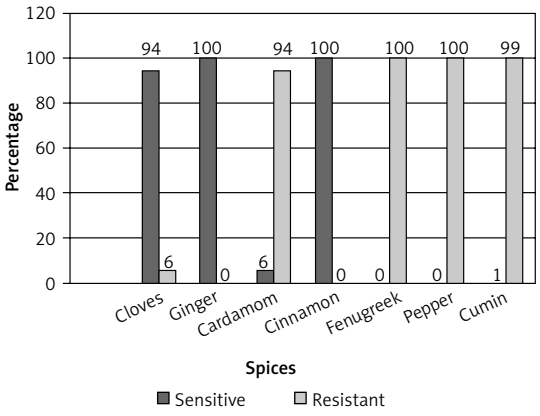

Figure 1. Antimicrobial activity of species (%)

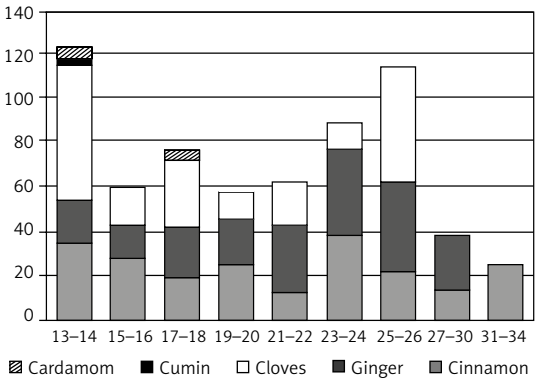

Figure 2. Zone diameter of the 7 spices for enterococci

Table IV. Antimicrobial sensitivity pattern of different spices for *E. faecalis* and *E. faecium*

Organism	Cinnamon	Ginger	Cloves	Cumin	Cardamom	Fenugreek	Pepper
E. faecalis (*n* = 102)	102 (100%)	102 (100%)	96 (94%)	1 (1%)	7 (7%)	0	0
E. faecium (*n* = 42)	42 (100%)	42 (100%)	40 (97%)	1 (1%)	1 (4%)	0	0

Table V. Antimicrobial sensitivity pattern of different spices in unusual enterococcal species

Organism	Cinnamon	Ginger	Cloves	Cumin	Cardamom	Fenugreek	Pepper
E. avium (*n* = 2)	2 (100%)	2 (100%)	1 (50%)	0	0	0	0
E. durans (*n* = 1)	1 (100%)	1 (100%)	1 (100%)	0	0	0	0
E. raffinosus (*n* = 1)	1 (100%)	1 (100%)	1 (100%)	0	0	0	0

In this study we also analyzed the antimicrobial effect of cinnamon, cloves, ginger, cumin, black pepper, cardamom and fenugreek seeds. Cinnamon, ginger and cloves showed excellent antimicrobial activity against HLGR enterococci. Cardamom and cumin also showed sensitivity against a few isolates. Black pepper and fenugreek did not show any activity against the isolates. The isolates which showed sensitivity to the spices exhibited a zone diameter of inhibition (ZDI) greater than 7 mm. Cinnamon exhibited the best anti-enterococcal activity, producing ZDI ranging between 31 mm and 34 mm. By the agar diffusion method, the growth inhibitory activity was followed by the action of ginger with ZDI 25–30 mm; 25 (17%) were the most sensitive isolates for which the ZDI values were 27–30 mm. The maximum ZDI exhibited by cloves was 25–26 mm by 52 isolates and by cardamom was 17–18 mm. The isolates showed less sensitivity to Cinnamon (CMN); however, 2 (1%) of the tested isolates had ZDI 14 mm. Thus, the three spice extracts exhibited different degrees of growth inhibition against *enterococcal* isolates, and the ranking of anti-enterococcal activity of the extracts tested is cinnamon = ginger > cloves > cardamom > cumin.

Cinnamomum zeylanicum bark is rich in cinnamaldehyde (50.5%), which has been proven to be active against many pathogenic Gram-positive and Gram-negative bacteria [15, 16]. Ali *et al*. reported cinnamaldehyde as an active agent to inhibit the growth of both antibiotic-sensitive and -resistant strains of *Helicobacter pylori* [17]. It has been reported that *S. aromaticum* oil contains a high level (75%) of eugenol, and the antibacterial activity of *S. aromaticum* is attributed to this compound [15, 16]. Another important antimicrobial compound is tannin, in *S. aromaticum*, which also aids the process of antimicrobial action [17]. The antibacterial activity of *C. cyminum* essential oil is perhaps attributable to the high levels of cumin aldehyde (16.1%); the other main component is *α*-pinene (11.4%). Nanasombat reported that carvone and carvacrol also have inhibitory effects on bacterial strains [18]. Here, we have not studied the antibacterial activity of the components within the extracts; however, the compounds mentioned above may be responsible for the anti-enterococcal activity of the extracts, as has been investigated in our study.

Our study only analyzed the zone of inhibition as a parameter for the antibacterial activity of spices against the isolates. However, for an accurate description of the antimicrobial properties of the spices we suggest performing MIC and Kill kinetics (rate and extent of bacterial killing). Studies have shown that it displays better sensitivity trends to physicians than disc diffusion methods [16]. Time kill experiments should also be considered as a tool to determine whether the activity of the spices is dosage and time dependent so that it would imply a more rational basis for determining the optimal dosage of antimicrobial treatment regimens in order to combat the spread of antimicrobial resistance [16].

In conclusion, *C. zeylanicum* and *Z. officinale* showed the maximum antibacterial activity against the enterococcal isolates, followed by *S. aromaticum* and *C. cyminum*. The findings of the study show that spices used in the study can contribute to the development of potential antimicrobial agents for inclusion in the anti-enterococcal treatment.

Conflict of interest

The authors declare no conflict of interest.

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