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IN VITRO ANTIBACTERIAL ACTIVITY OF SEVEN SPICES AGAINST CLINICAL ISOLATES OF ENTEROCOCCI

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ABSTRACT

***Aim**: To explore the in vitro antibacterial activity of seven ethanolic extracts of spices against the clinical isolates of enterococci. **Methods**: 215 enterococcal strains were isolated from clinical samples. 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 well diffusion method. Statistical analysis was carried out by Chi-square test using SPSS 17 software. **Results**: Only Cinnamon and Ginger were found to have activity against all the isolates whereas Cumin and Cloves had varied 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 ranged from 31-34mm, 27-30mm, 25-26mm and 19-20mm respectively. **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 shows that spices used in the study can contribute in the development of potential antimicrobial agents for inclusion in the anti enterococcal treatment regime.*

KEY WORDS

Enterococci, antimicrobial sensitivity testing, ethanolic extracts of spices.

INTRODUCTION

Enterococci classified as Group D streptococci are the normal intestinal flora in humans. They are second only to Escherichia coli as agent of nosocomial UTI and third behind Staphylococcus aureus and Coagulase negative staphylococci as agents of nosocomial bacteremia.[1,2] Enterococcus faecalis is the species which dominate among the enterococcal human infections while majority of the remainder are caused by Enterococcus faecium.

A major contributing factor to pathogenesis of enterococci is their resistance to a wide variety of antibiotics. Enterococci have intrinsic resistance as well as can acquire resistance against a wide variety of antibiotics.

Antibiotic resistance has become a serious threat around the globe. This has forced mankind to search for newer safe and effective alternative from natural resources available on the earth and one such alternative is plant sources. Herbal extracts are gaining popularity in terms of natural antimicrobial preservatives or additives.[3] People of India as well as other parts of the world are using spices to enhance flavor and aroma of the foods. They have a long standing practice of using them in the treatment of ailments.[158] Antibacterial activities of extracts of different plants against various microorganisms

have been reported by many scientists.[4,5] Some medicinal herbs have also been assessed. Some spices were specifically tested for anti-microbial activities.[6]However there are scanty reports of the antimicrobial activity of the various Indian kitchen spices specifically against multi drug resistant clinical isolates of enterococci possessing diverse genetic machinery of virulent factors. Many studies have only dealt with standard strains of enterococci but not the clinical isolates.[7,8]

The present study aims at evaluating the effect of seven spices which are commonly used in Indian cuisine on different species of enterococci obtained from divergent clinical manifestations. If proved of value they can be systematically screened for any novel active compounds.

MATERIALS AND METHODS

A) Enterococcal isolates:

A total of 215 enterococci strains were isolated from different clinical samples received in the Department of Microbiology of a tertiary care center. They were speciated in accordance to a biochemical key as given by Facklam and Collins.[9]

B) Spices:

Seven types of typical Indian spices and herbs namely Piper nigrum (black pepper , Cuminumcyminum (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, seed and rhizomes barks, and buds were purchased from a local retail market. All the spices were washed thoroughly with distilled water to make them completely free from any contaminated particulate matter. The spices were then air dried and used for further procedure.

1) Extraction of spices:

The extraction of spices was carried out according to a procedure given by Manoj Kumar Singh *et al* which is as follows.[10]

a) Preparation of the powdered spices:

The air dried materials were powdered using a mixer grinder.

b) Soxhlet extraction:

150g of the powdered plant material was subjected to Soxhlet extraction using ethanol (500mL) as an extracting solvent. The extraction temperature was 78^0C. The time of extraction was 3-6 hrs or till 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 4^0C until use.

2) Preparation of the working solution:

The crude extract was dissolve in dimethyl sulphoxide (DMSO) (10-40%) by dissolving 1gm extract in 10ml of it. The concentration of the stock solution becomes 100mg/ml. The solutions were stored in refrigerator.

3) Inoculum preparation:

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

4) Evaluation of the antibacterial activity of the spices extract:

The crude extracts of spices were screened for its antimicrobial activity against the organisms by agar well diffusion method given by Dingle *et al.*[11] Sterile cotton swab was dipped in to the prepared inoculums and seeded all over the MHA 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 few minutes at room temperature with lid closed. Then with the help of sterile cork borer (6mm), wells were made in the inoculated plate and labeled. 50µl of the working suspensions of the spices extract were dispensed in the respective wells with the help of the micropipette. The solvent DMSO itself was tested for its activity as a control as 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 hr then observed for the zone of inhibition which is suggested by the clear area around the well.

Statistical analysis

The experiments were done at least twice and their mean values were represented. All statistical analyses including Chi square and Fischer's exact test were done in SPSS Version 17.0. Differences were considered significant when $p< 0.05$.

RESULTS

The 215 isolates of enterococci were subjected to a series of biochemical tests as per the key given by Facklam and Collins the result of which is as depicted in the following table.

Table 1 – Percentage distribution of various species of enterococci isolated from clinical samples.

Enterococci species isolated n = 215	Number of isolates (%)
E. faecalis	113 (58%)
E. faecium	70 (33%)
E. avium	11 (5%)
E. raffinosus	8 (4%)
E. casseliflavus	5 (2%)
E. durans	6 (3%)
E. gallinarum	2 (1%)

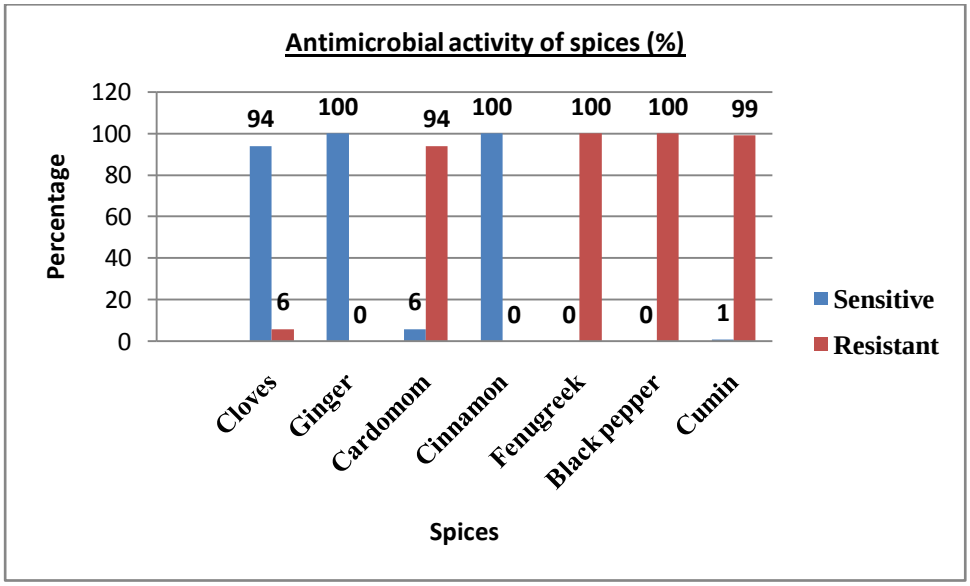
The spice extracts were obtained using Soxhlet apparatus and the physical characteristics of the same studied which is presented in the table given below.

Table 2: Physical characteristics of the spice extract

Extract	Color	Texture	Solubility	Smell
Black pepper	Black	Oily	DMSO	Spicy
Cardamom	Green	Oily	DMSO	Spicy
Cinnamon	Red	Oily	DMSO	Spicy
Fenugreek	Yellow	Oily	DMSO	Spicy
Cloves	Brown	Gummy	DMSO	Spicy
Ginger	Yellowish brown	Gummy	DMSO	Spicy
Cumin	Brown	Watery	DMSO	Spicy

The antimicrobial activity of the above seven spices was tested against the multi drug enterococcus by using agar well diffusion assay. The comparative bioactivity of the spices is as shown in the **Fig 1**

Fig 2: Antimicrobial activity of the seven spices used in the study.



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 above figure. (**Fig 2**)

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

Fig 3: Zone diameter of the seven spices for the enterococcal isolates.

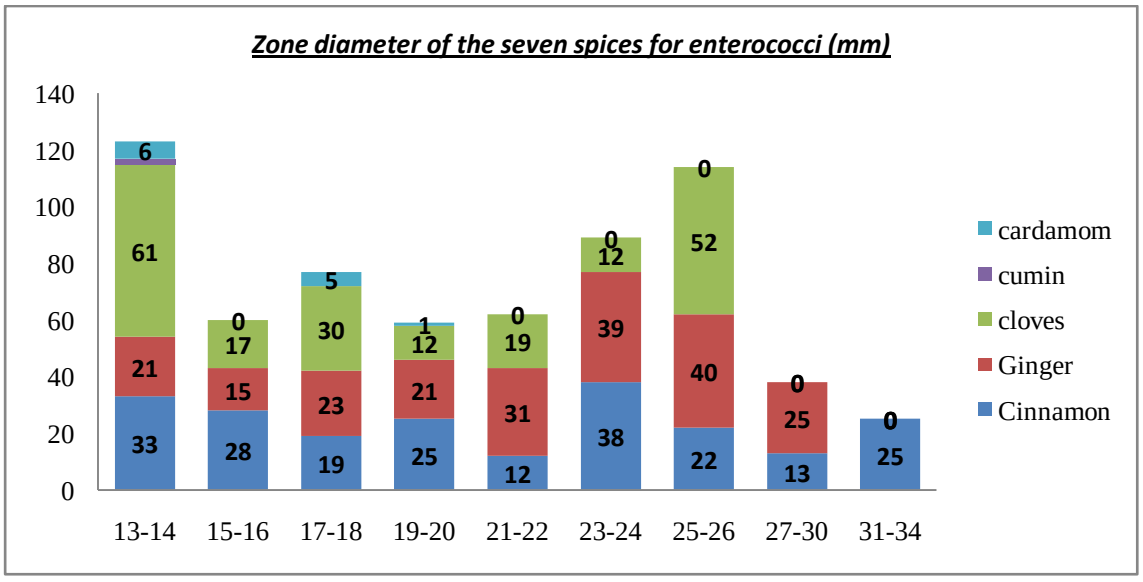


Table 2: Antimicrobial sensitivity pattern of different spices for *E.faecalis* and *E.faecium*.

Organism	Cinnamon	Ginger	Cloves	Cumin	Cardamom	Fenugreek	Pepper
E.faecalis n=113	113(100%)	113(100%)	106(94%)	1(1%)	8 (7%)	0	0
E.faecium n=70	70 (100%)	70 (100%)	68 (97%)	1(1%)	3 (4%)	0	0

Table 3: Antimicrobial sensitivity pattern of different spices on unusual enterococcal species.

Organism	Cinnamon	Ginger	Cloves	Cumin	Cardamom
E.avium n=11	11 (100%)	11(100%)	9 (82%)	0	0
E.casseliflavus n=5	5 (100%)	5 (100%)	5(100%)	0	0
E.gallinarum n=2	2 (100%)	2 (100%)	2(100%)	0	1 (50%)
E.durans n=6	6 (100%)	6 (100%)	6(100%)	0	0
E.raffinosus n=8	8 (100%)	8 (100%)	8(100%)	0	0

The above table shows that all the different species of enterococcus was maximally inhibited by cinnamon and ginger. Cumin had an inhibitory effect only on one isolates of E.faecalis eight (7%) and E.faecium 3 (4%) each, whereas cardamom showed an antibacterial activity against *E.faecalis, E.faecium* and one (50%) *E.gallinarum.*

Statistical analysis:

The χ^2-test was employed to compare the antibacterial activities of all the spices, The significant differences in activity were found between cinnamon and clove (p=0.03), as well as between Cinnamon and cardamom (p=0.020). Similarly 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. As the resistant strains develop ways around our traditional methods, it may be important to look into alternative methods to fight back. The alternatives may be as simple as looking in your spice cabinet.

With this aim we tested antimicrobial activity of seven spices against the multi drug clinical isolates of enterococci. From the early stages of synthetic antibiotic use, bacteria were already evading our efforts. Bacteria become resistant by actually destroying the drug, some staphylococci create penicillinase, which destroys penicillin. Some bacterial sites where the antibiotic attaches are altered making them less susceptible to the drug.[12] The overuse of antibiotics and the rate that bacteria replicate make the antibiotics less effective, which in turn creates more resistant strains.

Because of this bacterial behavior, it has been important for scientists to conduct research that will aid them in changing antibiotics to increase their effectiveness. The most promising spices that displayed antibacterial properties according

to this study are cinnamon and ginger which showed antimicrobial effect on all the isolates while cloves cardamom and cumin showed activity against some isolates. It could also be said that the concentration makes a difference.

The *C. zeylanicum* bark is rich in cinnamaldehyde (50.5%), which has been proven to be active against many pathogenic gram-positive and gram-negative bacteria.[13] Ali et al. reported cinnamaldehyde as the active agent to inhibit the growth of both antibiotic-sensitive and -resistant strains of *Helicobacter pylori*.[14] It has been reported that *S. aromaticum* oil contains high (75%) eugenol, and the antibacterial activity of *S. aromaticum* is attributed to this compound.[13] Another important antimicrobial compound is tannin, in *S. aromaticum,* which also aids the process of antimicrobial action. The antibacterial activity of *C. cyminum* essential oil is perhaps attributable to the high levels of cumin aldehyde (16.1%), the other main component includes *α*-pinene (11.4%).[15, 16]. 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.

Many studies have reported the antibacterial activity of different spices against a broad range of Gram positive and Gram negative strains. In all these studies a standard strain (*E.faecalis ATCC 15753*) of enterococci along with other microorganisms were tested for its susceptibility and resistance against the spices.[8,10] Several studies have reported that cinnamon have shown a significant antibacterial activity against the standard strain which is similar to our study with enterococcal isolates.[17,18,19] Some studies have also reported equal or high broad spectrum antimicrobial activity of the spices when compared with standard antibiotics tested.[10]

Our study only analysed the zone of inhibition as a parameter for the antibacterial activity of spices against the isolates. However for accurate description of the antimicrobial property of the spices we suggest MIC and Kill kinetics (rate and extent of bacterial killing) to be done. Studies have shown it displays a better sensitivity trends to physicians than disc diffusion methods.[13] Time kill experiments should also be considered as 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.[13]

CONCLUSION

The degree of antibacterial activity of the spices tested can be represented in the following order: Cinnamon = Ginger > Cloves > Cardamom > Cumin. All the five spices were found to be excellent bactericidal agents. Thus, the above five spices could be selected for use as potential anti-enterococcal agents, which will also be of great benefit in combating antibiotic resistance of enterococcus causing serious human infections as found in the current study. However, further studies are required to determine the toxicity and proper dose selection, but before that; these agents can easily be utilized topically. The potential of these spices can be further investigated into the field of pharmacology, phyto-chemistry or food chemistry for better drug discovery.

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