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Antibacterial activity of Indian cuisine spices against drug resistant pathogenic bacteria

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Abstract
The present study was performed to evaluate antibacterial activity of acetone and ethanol extracts of three selected Indian spices clove (*Syzygium aromaticum*), cinnamon (*Cinnamon Zeylanicum*), ajwain (*Trachyspermum ammi*) against drug resistant pathogenic bacteria *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*. Minimum inhibitory concentration was determined for effective extracts. Ethanolic and acetone extracts of Clove were most effective on all the tested bacteria followed by ethanol and acetone extract of Cinnamon. The lowest antibacterial activity was recorded with ethanol extract of ajwain. All the bacteria tested were found sensitive to the extracts of three spices with highest antibacterial activity of 25 and 22 mm recorded against *S.aureus and P.areuginosa* respectively. Among all the extracts tested, ethanolic extracts of clove expressed lowest MIC of 0.6% w/v.

Keywords: antibacterial activity, *Syzygium aromaticum*, *Cinnamomum zeylanicum*, *Trachyspermum ammi*, ethanol, acetone, drug resistance

Introduction
About 2500 plant species from over 1000 genera are utilized in India's indigenous system of medicine, demonstrating the country's long history of herb and herbal therapy [1]. Spices have antibacterial properties in addition to imparting flavor and harsh stimulation to foods [2, 3]. Antibacterial activity has been discovered in natural antimicrobial substances present in spices [4, 5]. Spices have been used in Indian cooking for centuries to enhance flavor as well as to treat infectious ailments in the home. For ages, plant-derived compounds including spices, fruits, and vegetable extracts have been utilized to preserve and extend the shelf life of foods [6]. Leaves (coriander, mint), buds (clove), bulbs (garlic, onion), and fruits (red chilli, black pepper), cinnamon stems, ginger rhizomes, and other plant parts [7] are examples of spices.

Despite the fact that dozens of antimicrobial compounds exist, bacteria have an incredible ability to build resistance to even the most effective antimicrobial chemical [8]. The focus of the search is on medicinal herbs, which may show to be the best non-antibiotic option [9, 10, 11]. Black pepper, clove, cinnamon, and turmeric extracts were found to have antibacterial properties against pathogenic germs [12].

Cinnamon (*Cinnamomum zeylanicum*)
Cinnamon bark is a popular spice with a mild aroma and a warming, pleasant flavor. Confectionary, liquors, medicines, and cosmetics all use it to flavor their products. It has been discovered that it aids diabetics in sugar digestion. It's astringent, stimulating, and carminative, and it can help with nausea and vomiting. Cinnamon leaf oil is frequently used in perfumes and cosmetics [13]. Cinnamon bark oil has antifungal effects.

Clove (*Syzygium aromaticum)*
It's used as a carminative, aromatic, and stimulant in medicine. Clove oil is used in medicine for its antiseptic and antibacterial characteristics, particularly in dentistry, oral and pharyngeal therapies. It's used in toothpaste and mouthwash formulations, as well as soaps and perfumes. It has also been claimed that it aids diabetics in sugar absorption [13].

Ajwain
Thymol, an active component of ajwain seeds, is a powerful fungicide and germicide. It's one of the few spices that may offer flavour while also being beneficial to your health. It works well for common colds, earaches, and toothaches. Traditional medicine systems have long employed ajwain for a number of medicinal and pharmacological purposes [14]. In order to treat diseased conditions and rectify aural weakness, Persian practitioners used an eye and eardrop made from ajwain seeds [15]. Cough, pleurisy, and dysphonia have all been reported to be helped by ajwain [16].

Despite the fact that pharm companies have developed a number of novel antibiotics in the previous three decades, microbes' resistance to these medications has increased. Bacteria, in general, have the genetic potential to transfer and acquire drug resistance, which is used as a therapeutic agent [17].

Considering the adaptable behavior of bacteria and the drawbacks of typical antibiotics, such as effects on normal flora and allergies [18], the current study was undertaken with the goal of determining the antibacterial effect of spices towards antibiotic resistant strains. Spice was chosen as a substitute for two primary reasons:

1. Plants have been the model source of medicine since ancient times and 2. The increasing resistance to antibiotics and acceptance of herbal medicines by general population.

Materials and Methods

Test organisms

The organisms that have been employed as test in study are clinical isolates of pathogenic bacteria- *Escherichia coli*, *Staphylococcus aureus* and *Pseudomonas aeruginosa* which were obtained from the Princess Esra hospital, Hyderabad, India. The isolates were previously characterized [19] and identified by macroscopic, microscopic, biochemical methods [20] in the microbiology laboratory of the hospital and were stored on slants at 4^0C.

Preparation of bacterial inoculum

The intensity of the testing bacterial cultures was maintained to 0.5 McFarland standard (1.5 x 108 CFU/ml) for inoculum preparation by adding sterile distilled water. The clarity of bacterial suspension is adjusted using McFarland standards to keep the bacterial population within a specified range. 0.05 ml of barium chloride (BaCl2) (1.17 percent w/v BaCl2.2H2O) was mixed to 9.95 ml of 0.18M H2SO4 (1.0 percent w/v) with continual stirring to make the 0.5 McFarland standard. To reduce evaporation loss, the McFarland standards tube was firmly sealed and maintained for up to 6 months. The test and standard were evaluated on a white background with a distinct black line before being used [21].

Collection of spices

The spice cinnamon bark (*Cinnamomum zeylanicum*) ajwian seeds (*Trachyspermum ammi*) and clove (*Syzygium aromaticum*), were bought at Hyderabad's regional supermarket.

Preparation of extracts

The spices were initially dried in sunlight for two days and then in an oven at 40^0C for about 24 hours. Finally the dried materials were ground into fine powder in a grinder. Two extracts *viz*., acetone and ethanol (95%) extracts were prepared by dissolving 25 g of spices powder in 100 ml of solvents to make (25% w/v) extract and to minimise evaporation, the mixture was stored at room temperature for 24 hours in sterile screw cap bottles before being filtered using sterilised Whatman no.1 filter paper. The extract was filtered and then evaporated in a water bath until only 25 mL of extract remained in the container. The bactericidal activity of the ethanol and acetone extracts was tested using the agar well diffusion method.

Kirby-Bauer Disk Diffusion Susceptibility Test

The aim of the Kirby-Bauer susceptibility test [22] was to regulate the drug resistance/sensitivity of selected pathogenic bacteria. For this method Mueller Hinton Agar (MHA) plates were inoculated with 100µl of standardized inoculum ($1.5x10^8$ CFU/ml) of each selected bacterial culture, spread with sterile swabs and the before starting, plates were allowed to remain at room temperature for at least 3 to 5 minutes to allow the agar plate's surface to dry. to the next step. Seven commercially available standard antibiotic discs with a specified dose as per Clinical Laboratory Standard Institute, USA (CLSI-M100) belonging to five classes (Penicillin, Streptomycin, Tetracycline, Norfloxacin Ciprofloxacin, Gentamycin, and Erythromycin) were placed on the plate (Table-1) and the plates were kept in incubator in an inverted position at 37°C for 24 hours. Following incubation the diameter of inhibition zone DIZ was measured in mm. Bacteria were considered drug resistant based on the standard zone diameter breakpoints as per CLSI-M100 [23]. The bacteria were considered multi drug resistant if they were resistant to at least 3 different categories of antibiotics.

Table 1: Selected Class of Antibiotics for Testing Drug resistance on Pathogenic Bacteria.

S. No	Name of Antibiotic	Antibiotic Class
1.	Penicillin	β-Lactam
2.	Streptomycin	Aminoglycosides
3.	Gentamycin	
4.	Ciprofloxacin	Fluoroquinolones
5.	Norfloxacin	
6.	Tetracycline	Tetracyclines (4 ringed)
7.	Erythromycin	Macrolides

Determination of antibacterial activity

The antibacterial activity of six crude extracts (acetone and ethanol) of 3 spices against three pathogenic drug resistant bacteria The agar well diffusion technique [24] was used to assess the results. MHA plates were infected (in triplicates) with 100µl of standardised inoculum (1.5x108 CFU/ml) of each chosen bacterium and dispersed using sterile swabs. (CLSI-M02-A12) [25]. Wells or cups of 6 mm size were made with sterile borer into agar plates containing the bacterial inoculum. 100µl volume of the spice extract was poured into a well of inoculated plates. Solvents acetone and ethanol were used as positice control and DMSO (dimethyl sulfoxide) as negative control. The plates prepared were kept at room temperature for ten minutes permiting the diffusion of the extracts into the agar. After incubation for 24hrs at 37°C, the plates were observed.

An inhibitory zone surrounding the well holding the spice extract, indicates the existence of antibacterial action.

The inhibitory zone diameter (DIZ) has been measured and reported in millimetres millimeters. The average diameter of the inhibitory zone was computed

Determination of minimum inhibitory concentration (MIC)

The minimum inhibitory concentration (MIC) is defined as the lowest concentration of the antimicrobial agent that will inhibit the visible growth of a microorganism after overnight incubation.

The MIC was performed taking the extract which has expressed high activity against drug resistant pathogenic bacteria by broth dilution method [21].

The test was carried by adding acetone and ethanolic extracts in Mueller Hinton Broth (MHB) (2ml) at concentrations ranging from 0.2-10 % (w/v), inoculated with 100µl of inoculum size test organism. A control tube containing MHB and 100µl of test bacterial cultures (1.5 x 10^8 CFU/ml) was incubated for 24 hrs at 37°C.

Statistical analysis

All studies were repeated three times, mean values were reported in antibiotic resistance results, and Mean ± Standard Deviation values for antibacterial activity and MIC results.

Results and Discussion

In the present study, antibacterial activity of three selected Indian spices were tested against drug resistant bacterial

strains of *E.coli*, *S.aureus* and *P.aeruginosa*. Identification characteristics of the strains (data not shown) were previously confirmed with those of the standard manuals as mentioned in materials and methods. Drug resistance of selected pathogenic bacteria was determined by Kirby-Bauer disk diffusion susceptibility test method against commercially available antibiotics Penicillin, Streptomycin, Tetracycline, Norfloxacin, Ciprofloxacin, Gentamycin, Erythromycin, by comparing the measured DIZ values with that of standard breakpoints given by CLSI-M100 [23] (Table-2). Based on the results obtained, it was observed that *E.coli* strain was resistant to five antibiotics- penicillin, streptomycin, gentamycin, norfloxacin and ciprofloxacin and sensitive to two antibiotics-tetracycline and erythromycin, with a zone diameter in the range of CLSI breakpoints. *S.aureus* strain was found to be resistant to two antibiotics-penicillin and tetracycline, intermediate to ciprofloxacin and sensitive to four antibiotics- streptomycin, gentamycin, norfloxacin and erythromycin. *P.aeruginosa* strain was resistant to five antibiotics-penicillin, streptomycin, gentamycin, ciprofloxacin and norfloxacin, sensitive to one antibiotic-tetracyline and non-conclusive for one antibiotic-erythromycin due to non-availability of standard CLSI breakpoint.

The overall picture of drug resistance reveals that, all the three tested strains were multi drug resistant. Two strains *E.coli* and *P.aeruginosa* were resistant to aminoglycoside group and fluroquinolones group of antibiotics and sensitive to tetracycline and macrolides group of antibiotics; one strain *S.aureus* was resistant to only β-lactam and tetracycline group of antibiotics and was sensitive to aminoglycoside group, fluroquinolones group and macrolides group.

Table 2: Standard Zone diameter Breakpoints of Selected Antibiotics As per CLSI-M100

Bacteria	Antibiotic	Dose	Zone Diameter Breakpoint (mm)		
			S	I	R
E.coli	Penicillin	10µg	≥17	14-16	≤13
	Streptomycin	10µg	≥15	12-14	≤11
	Tetracycline	30µg	≥15	12-14	≤11
	Norfloxacin	10µg	≥17	13-16	≤12
	Ciprofloxacin	5 µg	≥21	16-20	≤15
	Gentamycin	10µg	≥15	13-14	≤12
	Erythromycin	15µg	≥13	-	≤12
P.aeruginosa	Penicillin	100µg	≥21	15-20	≤14
	Streptomycin	10µg	≥15	13-14	≤12
	Tetracycline	30µg	≥16	13-15	≤12
	Norfloxacin	10µg	≥17	13-16	≤12
	Ciprofloxacin	5 µg	≥21	16-20	≤15
	Gentamycin	10µg	≥15	13-14	≤12
	Erythromycin	15µg	-	-	-
Staphylococcus spp.	Penicillin	10µg	≥29	-	≤28
	Streptomycin	10µg	≥15	13-14	≤12
	Tetracycline	30µg	≥19	15-18	≤14
	Norfloxacin	10µg	≥17	13-16	≤12
	Ciprofloxacin	5 µg	≥21	16-20	≤15
	Gentamycin	10µg	≥15	13-14	≤12
	Erythromycin	15µg	≥18	14-17	≤13

S- Sensitive; I- Intermediate; R-Resistant

Table 3: Drug Resistance of Tested Bacterial Strains on Standard Antibiotics

Antibiotic	*E.coli*		*S.aureus*		*P.aeruginosa*	
	Inhibition Zone in mm	Drug Resistance	Inhibition Zone in mm	Drug Resistance	Inhibition Zone in mm	Drug Resistance
Penicillin	13	R	24	R	14	R
Streptomycin	8	R	15	S	12	R
Gentamycin	10	R	24	S	12	R
Ciprofloxacin	8	R	17	I	15	R
Norfloxacin	10	R	30	S	12	R
Tetracycline	15	S	14	R	20	S
Erythromycin	18	S	25	S	12	NC

R-Resistant; S-Sensitive; I-Intermediate; NC-Non Conclusive

Antibacterial activity of acetone and ethanolic extracts of selected three spices- clove (*Syzygium aromaticum*), cinnamon (*Cinnamomum zeylanicum*), and ajwain (*Trachyspermum ammi)* were studied by agar well diffusion method for determining antibacterial potential. The extracts of all the three spices showed inhibitory activity against all the drug resistant pathogenic bacteria tested with the diameter of inhibition zone in the range from 8-25mm. The ethanolic extract of clove showed highest activity against all the drug resistant bacterial pathogens with DIZ values between 18-25mm. Maximum inhibitory effect was recorded for *P.aeruginosa* (25mm), followed by *E.coli* (22mm) and *S.aureus* (20mm) and these results are in close accordance with Ram kumar *et al*.,[26]. Results of acetone extract of clove also showed high activity with DIZ values between 18-22 mm, with maximum DIZ of 22 mm for *S.aureus* followed by *P.aeruginosa* (20mm) and *E.coli* (18mm) (Figure-1). Nassar *et al*. observed that GC-MS

analysis of clove oil extract revealed that the predominant compounds were eugenol acetate, eugenol, and caryo-phyllene, the latter two of which are known to have antibacterial and antifungal effects [27].

Next highest antibacterial activity was observed with ethanol extract of cinnamon with a DIZ values in the range of 12-14mm respectively (Figure-1). For *S.aures* DIZ of 14mm was recorded and for *E.coli*, and *P.aeruginosa* DIZ of 12 mm was recorded. These inhibitory zones were much better than the results reported by Masih Usha *et al*.,[28]. Similarly, acetone extract of cinnamon also revealed elevated DIZ values ranging between 8-15mm, which are higher than the data described by Masih Usha *et al*. [28]. Results of one Indian study [29] indicated that cinnamon have potent antimicrobial activities against *Bacillus subtilis*, *Escherichia coli* and *Pseudomonas* spp. It can be suggested that the antimicrobial efficacy of cinnamon is due to eugenol and cinnamal aldehyde.

Acetone extract of ajwain also expressed inhibitory effect with a zone of inhibition less in comparison to the extracts of cinnamon and clove. DIZ for *S.aureus* was found to be 12 mm which was highest, indicating its sensitivity to the extract.

Whereas moderate activity was recorded against *E.coli* and *P.aeruginosa* with DIZ of 8mm. Ethanol extract of ajwan produced zone of inhibition of 9 mm against *E.coli*, 8mm against *S. aureus* and 10mm against *P.aeruginosa* respectively (Figure-1). Ajwain's whole essential oil may include substantial levels of thymol or Carvacrol (phenolic compounds), which have been found to be either bactericidal or bacteriostatic actions directly proportional to the concentration [30]. They have anti-inflammatory [31] and antiviral [32] properties as well.

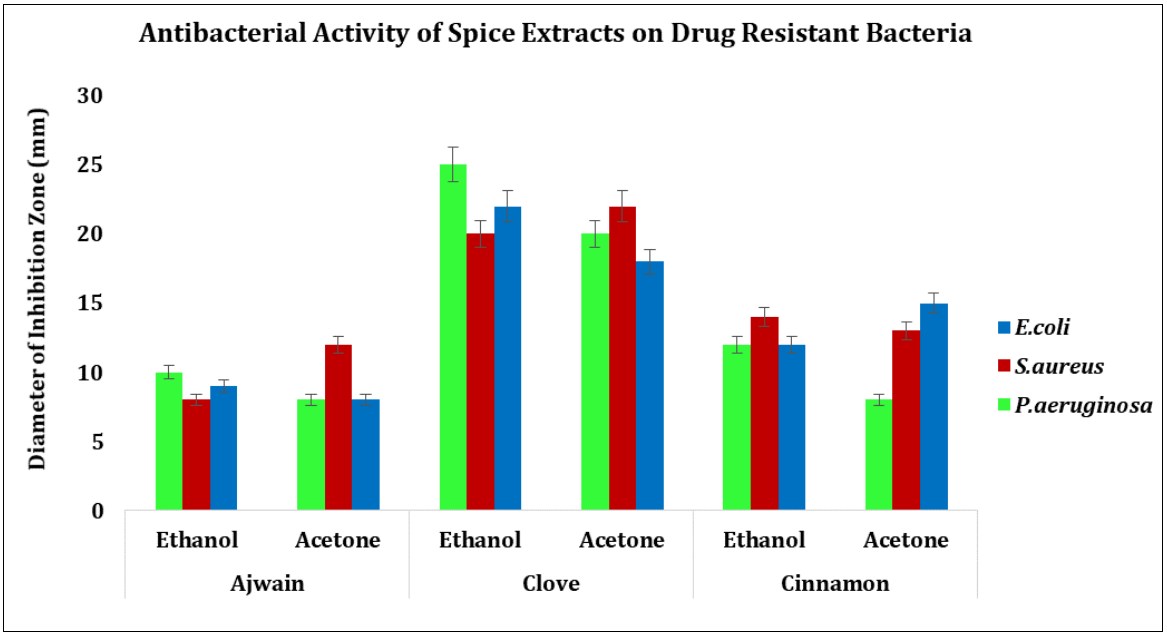

Fig 1: Antibacterial activity of ethanol and acetone spice extracts on drug resistant bacterial pathogens

Table 4: Minimum inhibitory concentration (MIC) of cinnamon acetone and ethanol extracts against drug resistant pathogenic bacteria

Bacteria	Acetone extract of cinnamon %w/v							
	0.2	**0.3**	**0.6**	**1.3**	**2.5**	**5**	**10**	**MIC**
E.coli	+	+	+	-	-	-	-	1.3
S.aureus	+	+	+	+	-	-	-	2.5
P.aeruginosa	+	+	+	+	+	+	-	10
	Ethanol extract of cinnamon %w/v							
	0.2	0.3	0.6	1.3	2.5	5	10	MIC
E.coli	+	+	+	-	-	-	-	1.3
S.aureus	+	+	+	+	-	-	-	2.5
P.aeruginosa	+	+	+	+	+	-	-	5

'+' = Growth formation, '-' = NO Growth Formation

Table 5: Minimum inhibitory concentration (MIC) of clove acetone and ethanol extracts against drug resistant pathogenic bacteria

Bacteria	Acetone extract of clove %w/v							
	0.2	**0.3**	**0.6**	**1.3**	**2.5**	**5**	**10**	**MIC**
E.coli	+	+	+	-	-	-	-	1.3
S.aureus	+	+	-	-	-	-	-	0.6
P.aeruginosa	+	+	+	-	-	-	-	1.3
	Ethanol extract of clove %w/v							
	0.2	0.3	0.6	1.3	2.5	5	10	MIC
E.coli	+	+	-	-	-	-	-	0.6
S.aureus	+	+	-	-	-	-	-	0.6
P.aeruginosa	+	+	+	-	-	-	-	1.3

'+' = Growth formation, '-' = NO Growth Formation

Inhibitory concentration at the bare minimum was carried out by broth dilution method for two spices cinnamon and clove out of three as ajwain showed least antibacterial effect by agar well diffusion method. MIC value of acetone and ethanol extracts of cinnamon was found to have similar activity for *E.coli* (1.3% w/v) and *S.aureus* (2.5% w/v) except for *P.aeruginosa*, where MIC was 10 % for acetone extract and (5%) for ethanol extract. (Table-4). Clove acetone extract was most effective in comparison to cinnamon extract with lower MIC values of 1.3 % against *E.coli* and *P.aeruginosa* and lowest MIC of 0.6% was recorded for *S.aureus*. On contrary clove ethanol extract exhibited maximum inhibitory activity with MIC value of (0.6% w/v) against *E.coli*, *S.aureus* and (1.3 %) for *P.aeruginosa* (Table 5). The overall picture of MIC of the two spice extracts is shown in Figure-2, which clearly indicates the high activity of clove ethanol extract at low concentration.

Antibacterial activity of acetone and ethanol extracts of cinnamon (*Cinnamomum zeylanicum*) and ajwain (*Trachyspermum ammi*) on four food spoilage bacteria was previously reported [28]. Antimicrobial resistance (AMR) is a growing concern to global public health that needs action from all levels of government and society. The safety of serious surgery and chemotherapy treatment would be jeopardised without efficient antibiotics [33]. Extensive research is being carried out to find an alternative to antibiotics, which can be used in various treatment procedures with the help of natural compounds [34]. Zaika [35] has reviewed the antimicrobial effectiveness of spices and herbs.

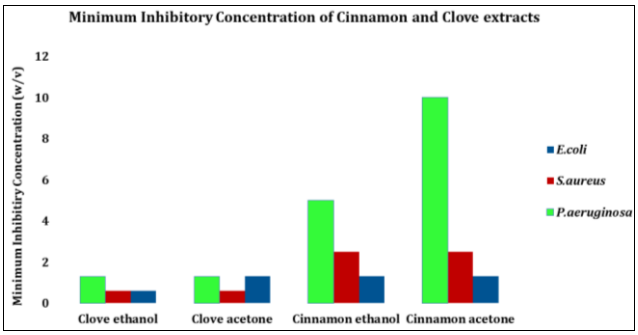

Fig 2: Minimum inhibitory concentration (MIC) of spice extract on drug resistant bacterial pathogens

Conclusion

Based on the results obtained in the present study we conclude that selected Indian spices has good antibacterial activity against drug resistant pathogenic bacterial clinical isolates- *E.coli*, *S.aureus* and *P.aeruginosa*. This gives a clue that these spices have the potential to be used in therapy as an alternative to antibiotics in future keeping in view the growing resistance to antibiotics. More research in this area is needed to extract, characterize and purify the active compounds and study their broad antibacterial spectrum against various pathogenic bacteria with clinical trials.

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