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Plasmid Borne Resistance in Bacteria to Common Household Spices

Anoor Pawan Kumar[1], Karthik Rajkumar[1], M. Swathi Raju[1], A. Nichita[1] and Sandeepta Burgula[1*]

[1]Department of Microbiology, Osmania University, Hyderabad 500007 Andhra Pradesh, India.

Authors' contributions

This work was carried out in collaboration between all authors. Author SB designed the study and edited the manuscript. Author APK performed major portion of the work and prepared the manuscript. Author KR performed a part of the experiments. Author s MSR, KR and AN managed the analyses of the study and helped with writing the manuscript and editing it. All authors read and approved the final manuscript.

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Original Research Article

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ABSTRACT

Aim: To examine the antimicrobial properties of various spices commonly used in Indian cuisine and to check if the resistance in bacteria is plasmid borne.
Study Design: To demonstrate the antimicrobial properties in various household spices like Cinnamon *(Cinnamomum zeylanicum)*, Clove *(Syzygium aromaticum),* Black Pepper *(Piper nigram)* and Turmeric *(Curcuma longa)* and to examine their effects on the pathogenic bacteria *Escherichia coli, Bacillus subtilis, Klebsiella pneumoniae, Pseudomonas aeruginosa* and *Staphylococcus aureus*.
Place and Duration of Study: Department of Microbiology, Osmania University, India during June 2013- January 2014.
Methodology: Antimicrobial assay was performed to determine the antimicrobial activity of these spices. Plasmids were isolated from bacteria showing resistance to these spices to confirm if the

**Corresponding author: Email: s_burgula@yahoo.com;*

resistance to these spices was plasmid borne. The isolated plasmids were used to transform *E. coli DH5α* which was previously observed to be sensitive to these spices.
Results: On transformation with plasmids isolated from *K. pneumoniae* and *P. aeruginosa*, *E. coli DH5α* cells were observed to gain resistance to all the four spices tested in the original strains of *K. pneumoniae* and *P. aeruginosa*.
Conclusion: The resistance to spices seems to be a plasmid borne feature which may be transferred to sensitive bacterial strains.

Keywords: Antimicrobial activity; cinnamon; pepper; turmeric; clove; plasmids; resistance.

1. INTRODUCTION

Spices are key ingredients in Indian cuisines this could be one reason why Indians are less prone to food borne infections. Bacteria are ubiquitous and continuously adapting to new environments. Even though antibiotics are effective in bacterial killing, microbes are continuously developing resistance to most antibiotics. In the last two decades, antibiotic resistance has become an emerging problem worldwide [1,2]. Multi-drug resistant strains of *E. coli* are widely distributed in hospitals and new strains are being identified every year [3]. For enterohemorrhagic *E. coli* 0157:H7 which is a food borne pathogen, the periplasmic catalase is encoded on the pO157 plasmid, and is believed to be involved in virulence by providing additional oxidative protection host infection [4]. Methicillin-resistant *S. aureus* (MRSA) is one of the greatly feared strains which have become resistant to most antibiotics. MRSA strains are most often found associated with institutions such as hospitals, but are becoming increasingly prevalent in the environment. The emergence of MRSA is only a general aspect of drug resistance. *Mycobacterium,* the genus harboring tuberculosis and leprosy strains, is a chronic menace in developing countries like India, where millions of patients acquire these infections every year. Mycobacterial infections are fastidious, in the sense that they require multiple drug therapy for a minimum of six months to two years [5,6]. Multiple drug resistance in Mycobacterium is prevalent worldwide and billions of dollars are spent every year to device effective treatment strategies against the pathogen. Bacterial plasmids are key vectors in horizontal gene transfer as they mediate the mobilization of gene from one bacterium to another and enable competent bacteria to take up the DNA and to spread within and between bacteria species by conjugation, which facilitates the rapid dissemination of resistance to antibiotic, through a bacterial population [7]. Most bacteria R-plasmid confer resistance to antibiotics by many mechanisms some of which includes (1) Producing enzymes capable of destroying or inactivating the antibiotic (2) Altering the target site receptor for the antibiotics to reduce or block its binding (3) Preventing the entry of the antibiotic into the bacterium and/or actively transporting the antibiotic out of the bacterium and (4) Modulating gene expression to produce more of the bacteria enzyme altered by the antibiotic [8]. Due to the continuous evolution of bacterial resistance to currently available antibiotics, it has become necessary to search for novel and effective antimicrobial compounds. Globally, plant extracts are employed for their antibacterial, antifungal and antiviral activities. It is known that more than 400,000 species of tropical flowering plants have medicinal properties and this has made traditional medicine reliable and cost effective on par with modern medicine [9]. Spices are essential components of Indian cuisines since ancient times. These are being used in minute amounts to impart flavor, taste and aroma in food preparation [10]. Many spices have potent antimicrobial properties [11]. Cinnamon is used as a spice and an aromatic. The bark or oil has been used to combat microorganisms, as an antimicrobial substance. The essential oil is primarily composed of 65% to 80% cinnamaldehyde and small amounts of other phenols and terpenes, including eugenol, trans-cinnamic acid, hydroxycinnamaldehyde, o-methoxycinnamaldehyde, cinnamyl alcohol and its acetate, limonene, α-terpineol, tannins, mucilage, oligomeric procyanidins, and trace amounts of coumarin. Clove is a dried flower bud and essential oils are obtained from the buds, stems, and leaves. The principal constituent of distilled Clove bud oil (60% to 90%) is eugenol (4-allyl-2-methoxyphenol). Eugenol is used in medicine as local antiseptics. The oil also contains about 10% acetyleugenol and small quantities of Gallic acid, terpenes, furfural, vanillin, and methyl-n-amyl ketone. Essential oils contain very complex mixtures of compounds, varied from monoterpenes to sesquiterpenes

[12]. Black pepper gets its spiciness due to the chemical piperine. Black pepper, either powdered or its decoction, is widely used in traditional Indian medicine and as a home remedy for relief from respiratory infections. Black pepper contains between 4.6% and 9.7% piperine by mass, the aroma of pepper is attributed to rotundone(3,4,5,6,7,8-Hexahydro-3α,8α-dimethyl-5α-(1-methylethenyl)azulene-1(2H)-one). In case of turmeric, its important chemical components are a group of compounds called curcuminoids, which include curcumin (diferuloylmethane), demethoxycurcumin, and bisdemethoxycurcumin. The best studied compound is curcumin, which constitutes 3.14% of powdered turmeric. Curcumin is believed to have a wide range of biological effects including anti-inflammatory, antioxidant, antitumor, antibacterial and antiviral activities, which indicate potential in clinical medicine [13].

The present study was planned to check the antimicrobial activity of these spices on some commonly found normal flora which are frequently opportunistic pathogens. Natural antimicrobials are receiving a great deal of attention for a number of micro-organism-control issues. Reducing the need for antibiotics, controlling microbial contamination in food, improving shelf-life, decreasing the development of antibiotic resistance by pathogenic microorganisms or strengthening immune cells in humans are some of the benefits of using such antimicrobials. Finding the most inhibitory spice depends on a number of factors such as type of spice, effects on organoleptic properties, composition, concentration and biological properties of the antimicrobial and the target micro-organism, processing and storage conditions of the spice [14].

2. MATERIALS AND METHODS

2.1 Collection of Materials and Samples

2.1.1 Plant materials

The spices Cinnamon *(Cinnamomum zeylanicum)*, Clove *(Syzygium aromaticum)*, Pepper *(Piper nigram)* and Turmeric *(Curcuma longa)* used in this study were of Agmark grade and were purchased from Heritage foods, a local grocery in Hyderabad, India.

2.1.2 Bacterial cultures

Pure cultures of five bacterial strains, namely *S. aureus* and *B. subtilis, E. coli*, *P. aeruginosa* and *K. pneumoniae* used in this study were collected from the Health Center at Osmania University; Hyderabad and biochemical tests were performed to confirm the identity of these strains. The biochemical tests include Indole, Methyl red, Voges Proskauer and Citrate Utilization. The purpose for selecting the above bacteria was that all of them are opportunistic pathogens commonly associated with bacterial infections [15,16].

2.1.3 Biochemical tests

2.1.3.1 Indole test

When bacteria grow, tryptophan present in the growth medium is partially degraded resulting in accumulation of indole. When 0.5 ml of kovac's reagent is added to overnight culture, formation of red color in the alcohol layer of kovac's reagent indicates a positive test for indole.

2.1.3.2 Methyl red test

Several bacteria ferment glucose and form acid to decrease pH of the medium to a pH value below 4.5 which is tested with methyl red indicator. Bright red color indicates positive test for acid production and yellow color indicates a negative result.

2.1.3.3 Voges proskauer test

Some bacteria ferment glucose but do not decrease pH as they do not form sufficient amount of acid. Instead 'acetyl methyl carbinol' or its reduction product '2, 3-butylene glycol' is formed which does not change pH. 1ml of 40% KOH and 3 ml of 5% α- naphthol in alcohol solution is added to 5 ml of bacterial culture grown overnight. Positive reaction is indicated by the development of pink color in 2-5 min which turns crimson in 30 min.

2.1.3.4 Citrate utilization test

When a bacterial species is grown on Simmon's citrate agar, if bromothymol blue indicator present in the medium changes from light green to blue, it indicates a positive reaction for citrate utilization.

2.2 Methanol Extraction

Spice extracts were prepared as mentioned previously to obtain their active components which are organic in nature. About 10 g of

powdered spice was extracted with a mixture of dichloromethane and methanol (1:1, v/v) (2 x 50 ml) under reflux for 30 min and filtered to isolate the active organic compounds. The filtrates were combined and dried using a rotary evaporator (Eppendorf, USA) and then stored at 4ºC until further use.

2.3 Extraction in Phosphate Buffered Saline (PBS)

The dried spices were washed thoroughly with sterile distilled water to remove any possible contaminants. Aqueous decoction of each spice was prepared by boiling 10 g of dry and powdered spice in 100 ml sterile PBS over moderate flame for 30 min. The aqueous extract was cooled, filtered through Whatman No.1 filter paper and then stored in sterile 50 ml Falcon tubes at 4ºC [17].

2.4 Preparation of Inoculums

All the cultures obtained were stored and maintained on nutrient agar slants at 4ºC until further use (up to 4 weeks).Nutrient agar used was purchased from (Hi-Media Laboratories Ltd., Mumbai, India). Before performing agar well assay, each of the cultures were inoculated onto nutrient broth and incubated overnight at 37ºC in order to obtain a rapidly growing culture.

2.5 Antibiotic Assay

Antibiotic assay was performed on each of the cultures using antibiotic discs containing antibiotic at the concentrations of 10, 30 and 50 µg/disc. The reason for choosing the following antibiotics was because these are the most common antibiotics which are prescribed by physicians in India (Table 2).

2.6 Antimicrobial Assay by Agar Well Diffusion

Mueller Hinton agar [18] (Hi-media Laboratories Ltd., Mumbai, India) (plates were prepared using 3.8 g of the powdered medium in 100 ml of water and autoclaving it at 15 lb pressure for 15 min. 15 ml each of this autoclaved media was poured into petri plates of 10 cm diameter and depth of 1.2 cm and was allowed to solidify. Then wells of 0.5 cm were cut using a well borer. In few plates the wells were filled with 50 µl of the concentrated extracts. A combination of two extracts were prepared in the ratio of 1:1 and the wells were then added with the 50 µl of the combined extract in the Mueller Hinton agar plates spread with different cultures and were incubated at 37ºC for 24 h. After incubation, zones of inhibition were measured. The experiments were carried out in triplicate independently to confirm uniformity of data.

2.7 Plasmid Isolation by Alkaline Lysis Method

Plasmid isolation was performed using alkaline hydrolysis method and the plasmids were isolated from each culture and run on 1% (w/v) agarose gel and checked under a gel documentation system (Bio-rad).

Aliquot of 1.5 ml of bacterial culture in Luria Bertani (LB) broth was taken in a micro centrifuge tube and centrifuged at 15000 X g for 3 min at 4ºC. The supernatant was discarded and pellet was resuspended in 150 µl of ice-cold Alkaline Lysis Solution 1 (50 mM Glucose, 25 mM Tris-Cl (pH 8.0), and 10 mM EDTA (pH 8.0)) and vortexed vigorously. 250 µl of Alkaline Lysis Solution 2 (10N NaOH, 10% SDS) was added and mixed gently, followed by addition of 250 µl of Alkaline Lysis Solution 3 (5 M potassium acetate, glacial acetic acid) and centrifuged at 15000 X g for 15 min at 4ºC. Supernatant containing plasmid DNA was collected in a fresh tube and plasmid DNA was precipitated by adding 2 X volumes of absolute ethanol. The mixture was incubated at -20ºC for 1 h followed by centrifugation at 15000 X g for 10 min at 4ºC. Supernatant was discarded and the pellet was rinsed with 70% ethanol and centrifuged at 15000 X g for 5 min at 4ºC. The pellet was air-dried and resuspended in 50 µl of sterile Diethylpyrocarbonate water and stored at -20ºC. The quantity of DNA was measured at 260 nm using a spectrophotometer (Elico, India).

2.8 Competent Cell Preparation

E. coli DH5α cells were grown from freshly streaked plate in 5 ml LB broth at 37ºC, under vigorous aeration for 24h. This 24h culture was inoculated into fresh broth (1/100 dilution). Cells were allowed to grow at 37ºC (250 rpm), until OD_{600} reached 0.4 (i.e~2-3 hours). The broth was rapidly chilled in ice bucket and cells were kept ice cold in all further steps. The cells were centrifuged for 10 min at 4ºC at 8000 X g and resuspended in half culture volume of ice-cold 0.1 M $CaCl_2$ solution and incubated on ice for 30 min. The cells were centrifuged for 10 min at 4ºC

at 8000 X g and resuspended by repeated pipeting in 1/10 culture volume of ice-cold 0.1 M $CaCl_2$ in 15% glycerol [19]. These cells were dispensed into 100 µl aliquots in micro centrifuge tubes and stored at -80ºC until further use. Each 100 µl aliquots contained 10^7-10^8 colony forming units.

2.9 Transformation

Transformation experiment was performed using the plasmids isolated by alkaline lysis method and the transformed culture was allowed to grow on media containing the antibiotic vancomycin with an MIC of 30 µg/ml as the selective factor. The DH5α competent cells were thawed on ice and ~ 5 µl of DNA was added to 100 µl of cells and mixed gently. The tubes were incubated on ice for about 30 min and subjected to heat shock at 42ºC in a water bath for 90-120 seconds. Tubes were replaced on ice for ~2 min and 900 µl of pre warmed LB (with no antibiotic added) was added and then incubated at 37ºC with gentle agitation for 45 min to 1 hr. Then 100 µl to 200 µl of this culture was spread on selective media (LB agar with vancomycin) and incubated at 37ºC overnight. Untransformed colonies cannot grow on these plates as they are sensitive to vancomycin. The transformed colonies were selected randomly and were grown overnight in flasks. Plasmids were extracted from randomly selected colonies by alkaline lysis and were checked for the presence of the transformed plasmid.

3. RESULTS AND DISCUSSION

3.1 Identification of Bacterial Strains

The identity of bacterial strains used in the study was confirmed by the biochemical tests performed for the individual microorganisms. The biochemical tests include Indole, Methyl red, Voges-proskauer and Citrate utilization (Table 1).

3.2 Antimicrobial Activity of Spices Extracted in Phosphate Buffered Saline (PBS)

Aqueous extracts of Cinnamon, Clove, Pepper and Turmeric were checked for their antimicrobial properties by well diffusion assay. In *E. coli* only Cinnamon showed a considerable amount of inhibition, whereas both Cinnamon and Clove showed prominent zones of inhibition against *B. subtilis*. None of the spices were found to affect the growth in *K. pneumoniae* and *P. aeruginosa*, while Cinnamon showed less activity compared to clove on *S. aureus (*Table 3).

3.3 Antimicrobial Activity of Spices Extracted in Organic Solvent

Among the selected spices, cinnamon exhibited inhibition against *E. coli*, *B. subtilis* and *S. aureus* whereas *K. pneumoniae* and *P. aeruginosa* were resistant. Clove showed inhibition against *E. coli*, *B. subtilis, K. pneumoniae* and *S. aureus* but not against *P. aeruginosa,* whereas pepper showed very little inhibition against *B. subtilis, K. pneumoniae* and *S. aureus.* No inhibition was observed against *E. coli and P. aeruginosa.* Turmeric exhibited slight inhibition against *E. coli, S. aureus* and *K. pneumoniae* but no inhibition against *P. aeruginosa.* All the spice extracts showed prominent zones of inhibition with cinnamon and clove (zone of inhibition up to 1 mm) (Fig. 1). Clove extracts showed greater antimicrobial activity among other spice extracts against *K. pneumoniae* whereas none of the spices were found to be effective against *P. aeruginosa* (Table 3).

3.4 Antimicrobial Activity by the Combination of Extracts in Organic Solvent

After checking for their individual activity, a combination of spices was checked for their combined activity by mixing the extracts of two spices together and then performing well diffusion assay on the pathogenic strains of *E. coli, B. subtilis, K. pneumoniae, P. aeruginosa* and *S. aureus*.

Combination of Cinnamon + Clove, Cinnamon + Turmeric and Cinnamon + Pepper showed pronounced zones of inhibition on *B. subtilis*, while Cinnamon + Clove and Cinnamon + Turmeric showed inhibition but Cinnamon + Pepper did not show any inhibition on *E. coli* and none of the three combinations of Cinnamon + Clove, Cinnamon + Turmeric and Cinnamon + Pepper were found to be inhibiting the growth of *K. pneumoniae, P. aeruginosa* and *S. aureus* (Table 4).

Table 1. Biochemical tests to confirm the identity of the bacterial strains collected from University Health Center, Osmania University

Biochemical tests	*E. coli*	*B. subtilis*	*K. pneumonia*	*P. aeruginosa*	*S. aureus*
Gram staining	-	+	-	-	+
Indole	+	-	-	-	-
Methyl red	+	-	-	-	+
Voges-proskauer	-	+	+	-	+
Citrate utilization	-	-	+	+	-

**+ indicates positive result whereas *– indicates negative result*

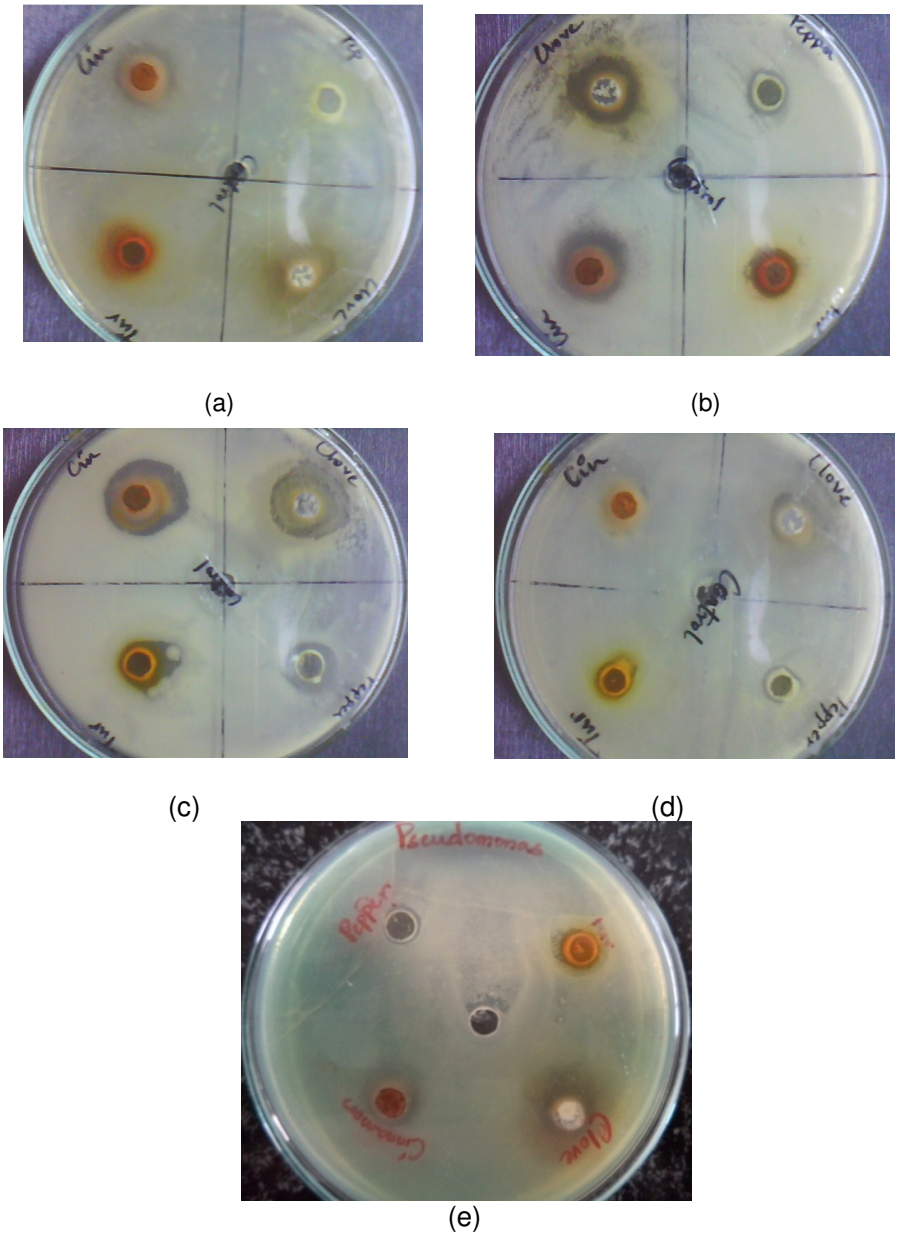

Fig. 1. Antimicrobial activity of clove, cinnamon, pepper and turmeric on (a) *E. coli*, (b) *S. aureus*, (c) *B. subtilis*, (d) *K. pneumoniae* and (e) *P. aeruginosa* using organic extraction method with absolute ethanol as control

3.5 Antimicrobial Activity by the Combination of Extracts

Combination of Clove + Turmeric, Clove + Pepper, Turmeric + Pepper were checked for their inhibitory activity on pathogenic strains of *B. subtilis, E. coli, K. pneumoniae, P. aeruginosa and S. aureus.* All the three combinations of Clove + Turmeric, Clove + Pepper, Turmeric + Pepper were found to be equally effective against *E. coli,* whereas only Clove + Turmeric and Clove + Pepper were found to be inhibiting *B. subtilis.* Turmeric + Clove didn't show any inhibition.(Table 4) None of the three combinations of Clove + Turmeric, Clove + Pepper, Turmeric + Pepper were found to be inhibiting the growth in *K. pneumoniae* and *P. aeruginosa* and only combination of Turmeric + Pepper was found to be inhibiting *S. aureus* (Table 5).

3.6 Plasmid Isolation

It was observed that the *E. coli* culture used did not contain any plasmid whereas *B. subtilis* and *S. aureus* had plasmids with a small copy number. *K. pneumoniae* and *P. aeruginosa* had a moderate copy number of their plasmid. *B. subtilis* and *S. aureus* had low copy number i.e.1.7 µg and 1.4 µg of DNA per ml of LB culture*. K. pneumoniae* had 2.6 µg of DNA per ml and *P. aeruginosa* had 1.9 µg of DNA per ml of LB culture indicating high copy number among the micro-organisms. Hence high copy number of resistance plasmid present in a bacterial cell showed higher resistance of the bacteria [20] (Fig. 2).

3.7 Transformation

It was found that colonies 2 and 3 of *K. pneumoniae* and colonies 2, 3 and 4 of *P. aeruginosa* were successfully transformed due to their growth in presence of vancomycin used as selective agent (Fig 3).

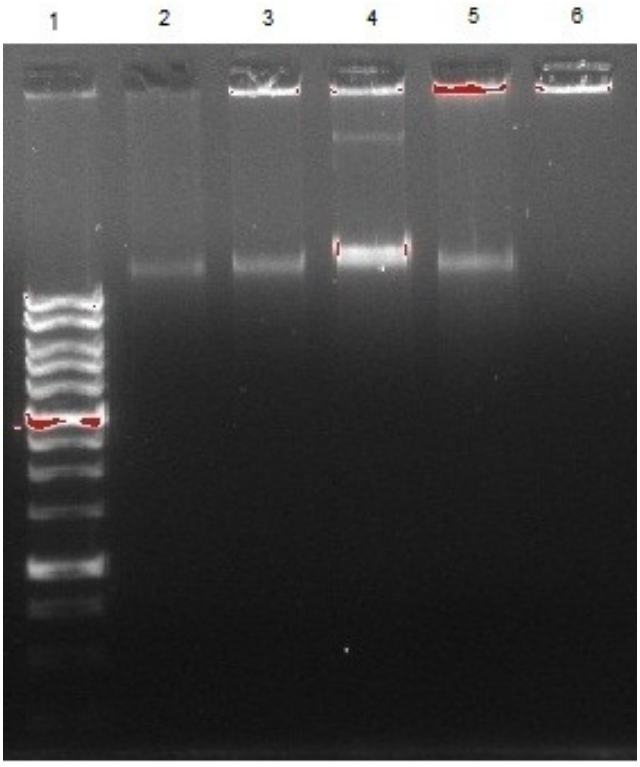

Fig. 2. Plasmid isolated from the 5 test organisms. Lane 1: 1kb marker, Lane 2: *S. aureus,* Lane 3*: B. subtilis,* Lane 4: *K. pneumoniae,* Lane 5*: P. aeruginosa,* Lane 6*: E. coli*

Table 2. Antibiotic assay using disc diffusion method

Antibiotic (µg/disc)	*E. coli (mm)*	*B. subtilis (mm)*	*K. pneumonia (mm)*	*P. aeruginosa (mm)*	*S. aureus (mm)*
Bacitracin (10)	-	1 ± 0.1	-	-	2 ± 0.1
Chloramphenicol (30)	3.4 ± 0.2	1.7 ± 0.1	2.2 ± 0.2	1.5 ± 0.1	3.2 ± 0.2
Gentamycin (50)	2.8 ± 0.2	2.2 ± 0.2	1.7 ± 0.1	3.4 ± 0.2	2.4 ± 0.1
Kanamycin (30)	2.5 ± 0.1	2 ± 0.2	1.8 ± 0.1	1.6 ± 0.1	2.4 ± 0.1
Methicillin (10)	-	-	-	-	2.7 ± 0.1
Streptomycin (10)	2 ± 0.1	1.8±0.1	1.5 ± 0.1	2.4 ± 0.1	2.2 ± 0.1
Tetracyclin (30)	2.5 ± 0.1	2.1 ± 0.2	1.8 ± 0.1	0.9 ± 0.1	3.2 ± 0.2
Tobromycin (10)	2.3 ± 0.1	2.2 ± 0.2	1.5 ± 0.1	1.7 ± 0.1	3.2 ± 0.1
Vancomycin (30)	1.1 ±0.1	1.6 ±0.1	-	-	1.4 ±0.1

** - indicates no inhibition.*mm indicates size of the zone in millimeters*

Table 3. Antimicrobial activity of spice extracts of cinnamon, clove, pepper and turmeric by organic and aqueous extraction method on the pathogenic strains

	E. coli (mm)		*B. subtilis (mm)*		*K. pneumonia (mm)*		*P. aeruginosa (mm)*		*S. aureus (mm)*	
	O	**A**	**O**	**A**	**O**	**A**	**O**	**A**	**O**	**A**
Cinnamon	0.5 ± 0.1	0.1± 0.1	1 ± 0.1	0.6 ± 0.1	-	-	-	-	0.2 ± 0.1	0.2 ± 0.1
Clove	0.3 ± 0.1	-	1 ± 0.2	0.6 ± 0.1	0.3 ± 0.1	-	-	-	0.4 ± 0.1	0.6 ± 0.1
Pepper	-	-	0.6 ± 0.1	-	0.1 ± 0.1	-	-	-	0.2 ± 0.1	-
Turmeric	0.1 ± 0.1	-	0.7 ± 0.1	-	0.1 ± 0.1	-	-	-	0.3 ± 0.1	-

**O indicates organic extract and A indicates aqueous extract. * - indicates no inhibition.*mm indicates size of the zone in millimeters*

Table 4. Antimicrobial activity by the combination of extracts of cinnamon + clove, cinnamon + turmeric and cinnamon + pepper in organic solvent

Combination	*E. coli (mm)*	*B. subtilis (mm)*	*K. pneumonia (mm)*	*P. aeruginosa (mm)*	*S. aureus (mm)*
Cinnamon + Clove	1.6 ± 0.1	2.0 ± 0.2	-	-	-
Cinnamon + Turmeric	1.5 ± 0.1	1.8 ± 0.1	-	-	-
Cinnamon + Pepper	-	1.5 ± 0.1	-	-	-

** - indicates no inhibition.*mm indicates size of the zone in millimeters*

Table 5. Antimicrobial activity by the combination of spices clove + turmeric, clove + pepper and turmeric + pepper

Combination	*E. coli (mm)*	*B. subtilis (mm)*	*K. pneumonia (mm)*	*P. aeruginosa (mm)*	*S. aureus (mm)*
Clove + Turmeric	1.5 ± 0.1	1.2 ± 0.1	-	-	-
Clove + Pepper	1 ± 0.1	1.2 ± 0.1	-	-	-
Turmeric + Pepper	1.5 ± 0.2	-	-	-	1.2 ± 0.1

** - indicates no inhibition.*mm indicates size of the zone in millimeters*

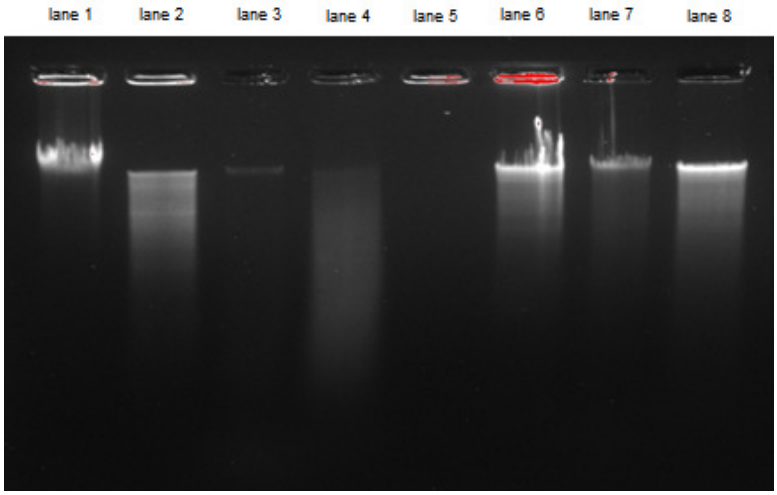

Fig. 3. Plasmids isolated from transformed colonies of *E. coli* DH5-α transformed using plasmids obtained from *K. pneumoniae* (Lane 1-4) and *P. aeruginosa* (lane 5-8)

Table 6. Antimicrobial activity of spices on the transformed colonies of *E. coli DH5α* where transformation was performed using plasmid isolated from *P. aeruginosa* and *K. pneumonia*

Spice	Control	Transformed by			
	E. coli DH5α (mm)	*P. aeruginosa* plasmid		*K. pneumoniae* plasmid	
		Colony 1 (mm)	Colony 2 (mm)	Colony 2 (mm)	Colony 3 (mm)
Cinnamon	1.3 ± 0.2	1.5 ± 0.1	-	-	1.5 ± 0.1
Clove	-	1.4 ± 0.2	2.1 ± 0.1	1.5 ± 0.1	1.7 ± 0.1
Pepper	-	-	-	-	-
Turmeric	1 ± 0.1	1.2 ± 0.1	1.6 ± 0.2	1.1 ± 0.1	1 ± 0.2

** –ve indicates no-inhibition.*mm indicates size of the inhibition zone in millimeters*
**E. coli DH5α was used as control*

3.8 Antimicrobial Activity of Spices on the Transformed Colonies

Antimicrobial activities of the spices were checked using well diffusion assay. It was found that *E. coli* DH5α which was initially sensitive to Cinnamon became resistant to it, whereas the cells were resistant to clove before transformation but became sensitive to it after transformation. This may be because both the resistance and sensitive genes were present on the same plasmid. Resistance to pepper and turmeric remained the same before and after transformation probably because resistance to these two spices was not present on the plasmid but might have been a natural phenomenon. It showed prominent zones of inhibition on the control culture. Cultures transformed using *P. aeruginosa* plasmids were inhibited by Cinnamon, Clove and Turmeric, while cultures transformed using *K. pneumoniae* plasmid were inhibited by only Clove and Turmeric indicating that plasmids obtained from *P. aeruginosa* carry genes of antimicrobial resistance which can be easily transferred to competent cells leading to spread of resistance to sensitive strains (Table 6).

4. DISCUSSION

Spices have been one of the most commonly used natural antimicrobial agents in foods and have been used traditionally for thousands of years by many cultures for preserving foods, to enhance aroma and to impart flavour [21]. Spices are considered as rich source of bio-active antimicrobial compounds [22]. Numerous studies have been published on the antimicrobial activities of plant extracts against different types of microbes, including food borne pathogens [23-25]. It has been reported that spices owe their antimicrobial properties mostly to the presence of alkaloids, phenols, glycosides, steroids, essential

oils, coumarins and tannins [26]. Besides, the use of synthetic compounds have significant drawbacks, such as increasing cost, handling hazards, concerns about residues on food and threat to human environment [27]. Extraction of spices with methanol and dichloromethane resulted in a product with greater antimicrobial activity than extraction with PBS, as aqueous extracts of all the spices displayed little or no antimicrobial activity against any of the bacteria tested. This is probably due to the fact that, though the solvents were removed from extracts by evaporation, different chemical compounds were extracted using various solvents, and most of the components with antimicrobial properties are aromatic or saturated organic compounds which are generally more soluble in solvents such as ethanol or methanol [28]. In a recent study on antimicrobial activity of Australian herbs; water, ethanol and hexane were used as the solvents for extraction, and it was observed that the aqueous extracts displayed little or no antimicrobial activity [29]. Many studies claim that phenolic substances present in spices may be responsible for antimicrobial activity [30]. Antibacterial activity of selected dietary spice was closely related to the concentration of phenolic compounds [31]. In this study, methanol and dichloromethane extracts of Cinnamon, Clove, Pepper and Turmeric proved to be more effective than the aqueous extracts of Cinnamon, Clove, Pepper and Turmeric.

Though many different herbs and spices were tested for their antimicrobial properties, fewer investigations have been carried out to study the synergistic effect of such substances. Our study indicated that spices showed antimicrobial properties but even greater antimicrobial activity was expressed by the combining the spices [32] because when cinnamon and clove were used individually they showed zones of inhibition in the range of 0.2 to 1mm as compared to combination of spices which showed increase in zones of inhibition from 1.5 to 2 mm in diameter. In general, Gram negative bacteria possessing plasmid showed greater resistance to the spices when used individually and in combined form [33]. Strongest antimicrobial effects were shown by cinnamon and cloves when *S. aureus, K. pneumoniae, E. coli, Enterococcus faecalis, Mycobacterium smegmatis, Micrococcus luteus*, and *Candida albicans* were used as test strains, showing inhibition zones between >10 and <30 mm by the disc-diffusion method [34]. Cinnamicaldehyde and eugenol extracted from cinnamon and clove at 0.03% and 0.04% concentrations showed antibacterial properties against *S. aureus. Bacillus sp.* was inhibited by cinnamic aldehyde and eugenol extracted from cinnamon and clove at just 0.1–1.0% w/v, 0.06% v/v concentration [35]. Gram positive bacteria are more susceptible towards the pepper extracts than gram negative bacteria. However, there are reports of occurrence of antibacterial activity against *S. aureus, E. coli, B. megaterium, B. sphaericus, B. polymyxa* [36,37]. Spices like turmeric showed maximum activity against gram positive when compared to gram negative bacteria individually and by mixtures [38]. *Pseudomonas*, specifically *P. aeruginosa* is the least sensitive group of bacteria to the action of essential oils and bioactive components of plant-origin [39].

K. pneumoniae organisms are often resistant to multiple antibiotics. Current evidence implicates plasmid as the source of the resistant genes [40]. *K. pneumoniae* with the ability to produce extended-spectrum beta-lactamases ESBL is resistant to many classes of antibiotics. The most frequent antibiotic resistances include resistance to aminoglycosides, fluoroquinolones, tetracyclines, chloramphenicol, and trimethoprim /sulfamethoxazole. *P. aeruginosa* is an opportunistic, nosocomial pathogen of immune compromised individuals. *P. aeruginosa* is naturally resistant to a large range of antibiotics. *P. aeruginosa* easily acquires resistance either by mutation in chromosomally encoded genes or by the horizontal gene transfer of antibiotic resistance determinants. Development of multidrug resistance by *P. aeruginosa* isolates requires several different genetic events, including mutations and/or horizontal transfer of antibiotic resistance genes.

Resistance (R) plasmids, historically known as R-factors, contain genes that provide resistance against antibiotics or toxins. In the current study bacteria containing plasmid were identified by subjecting each of them to plasmid isolation and running on agarose gel to observe the plasmid. It was observed that two of the cultures *K. pneumoniae* and *P. aeruginosa* showing resistance to most of the antibiotics and also to the spice extracts displayed a high copy numberof their plasmids. The isolated plasmid was then transformed into *E. coli DH5α* to check if the presence of the plasmid was responsible for their resistance to the extracts.

5. CONCLUSION

Spices have antimicrobial properties which may be beneficial in diet, especially in combinations. Plasmid borne resistance to spices seems to be naturally present in some bacteria such as *P. aeruginosa* and *K. pneumoniae*. Hence it is possible to transfer the resistance to other bacterial species, including some opportunistic bacteria which might be of some significance in people who may be at risk of acquiring opportunistic infections.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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