

PLASMID BORNE RESISTANCE IN BACTERIA TO COMMON HOUSE HOLD SPICES

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 nigrum*) and Turmeric (*Curcuma longa*) and to examine their effects on the pathogenic bacteria *E. coli* (*Escherichia coli*), *B. subtilis* (*Bacillus subtilis*), *K. pneumoniae* (*Klebsiella pneumoniae*), *P. aeruginosa* (*Pseudomonas aeruginosa*) and *S. aureus* (*Staphylococcus aureus*).

Place and duration of study: Department of Microbiology, Osmania University, Hyderabad, India during June 2013- January 14.

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 resistance to these spices was plasmid borne. The isolated plasmids were used to transform *E. coli* DH5 α which was previously observed to be sensitive to these spices.

Results: On transformation with plasmids isolated from *K. pneumoniae* and *P. aeruginosa*, *E. coli* DH5 α cells were observed to be resistant to all the four spices tested in the original strains of bacteria.

Conclusion: The resistance to spices seems to be a plasmid borne feature which is easily transferred by transformation to sensitive bacterial strains.

Keywords: Antimicrobial activity, Cinnamon, Pepper, Turmeric, Clove, plasmids, resistance.

1. INTRODUCTION:

Spices are key ingredients in Indian cuisines this-which could be one reason why Indians are less prone to food borne infections. Since Bacteria are ubiquitous and continuously adapting to environments. Even though antibiotics are effective in bacterial killing, resistance is microbes are continuously developing in bacteria-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 newer strains are being identified every year [3]. For enterohemorrhagic *E. coli* O157: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

Comment [D1]: Please indicate the number and species of the isolated bacteria, and the % and species of the resistant bacteria.

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36 oxidative protection host infection [4]. Methicillin-resistant *S. aureus* (MRSA) is one of the greatly
37 feared strains which have become resistant to most antibiotics. MRSA strains are most often found
38 associated with institutions such as hospitals, but are becoming increasingly prevalent in the
39 environment. The emergence of MRSA is only a general aspect of drug resistance. *Mycobacterium*,
40 the genus harboring tuberculosis and leprosy strains, is a chronic menace in developing countries like
41 India, where millions of patients acquire these infections every year. Mycobacterial infections are
42 fastidious, in the sense that they require multiple drug therapy for a minimum of six months to two
43 years [5, 6]. Multiple drug resistance in *Mycobacterium* is prevalent worldwide and billions of dollars
44 are spent every year to devise effective treatment strategies against such pathogens. Bacterial
45 plasmids are key vectors in horizontal gene transfer as they mediate the mobilization of gene from
46 one bacterium to another and enable competent bacteria to take up the DNA and to spread within and
47 between bacteria species by conjugation, which facilitates the rapid dissemination of resistance to
48 antibiotic, through a bacterial population [7]. Most bacteria R-plasmid confer resistance to antibiotics
49 by many mechanisms, some of which includes (1) Producing enzymes capable of destroying or
50 inactivating the antibiotic; (2) Altering the target site receptor for the antibiotics to reduce or block its
51 binding; (3) Preventing the entry of the antibiotic into the bacterium and/or actively transporting the
52 antibiotic out of the bacterium; and (4) Modulating gene expression to produce more of the bacteria
53 enzyme altered by the antibiotic [8].

54 Due to the continuous evolution of bacterial resistance to currently available antibiotics, it has become
55 necessary to search for novel and effective antimicrobial compounds. Globally, plant extracts are
56 employed for their antibacterial, antifungal and antiviral activities. It is known that more than 400,000
57 species of tropical flowering plants have medicinal properties and this has made traditional medicine
58 reliable and cost effective on par with modern medicine [9]. Spices are essential components of
59 Indian cuisines since ancient times. These are being used in minute amounts to impart flavor,
60 taste and aroma during food preparation [10]. Many spices have potent antimicrobial properties
61 [11]. Cinnamon is used as a spice and an aromatic. The bark or oil has been used to combat
62 microorganisms, as an antimicrobial substance. The essential oil is primarily composed of 65% to
63 80% cinnamaldehyde and small amounts of other phenols and terpenes, including eugenol, trans-
64 cinnamic acid, hydroxyl-cinnamaldehyde, o-methoxycinnamaldehyde, cinnamyl alcohol and its
65 acetate, limonene, α -terpineol, tannins, mucilage, oligomeric procyanidins, and trace amounts of
66 coumarin. Clove is a dried flower bud and essential oils are obtained from the buds, stems, and
67 leaves. The principal constituent of distilled Clove bud oil (60% to 90%) is eugenol (4-allyl-2-
68 methoxyphenol). Eugenol is used in medicine as local antiseptics. The oil also contains about 10%
69 acetyleugenol and small quantities of gallic acid, terpenes, furfural, vanillin, and methyl-n-amyl ketone.
70 Essential oils contain very complex mixtures of compounds, varied from monoterpenes to
71 sesquiterpenes [12]. Black pepper gets its spiciness due to the chemical piperine. Black pepper,
72 either powdered or as decoction, is widely used in traditional Indian medicine and as a home remedy
73 for relief from respiratory infections. Black pepper contains between 4.6% and 9.7% piperine by mass,
74 the aroma of pepper is attributed to rotundone (3,4,5,6,7,8-Hexahydro-3 α ,8 α -dimethyl-5 α -(1-
75 methylethenyl)azulene-1(2H)-one). In case of turmeric, its important chemical components are a group

76 of compounds called curcuminoids, which include curcumin (diferuloylmethane), demethoxycurcumin,
77 and bisdemethoxycurcumin. The best studied compound is curcumin, which constitutes 3.14% of
78 powdered turmeric. Curcumin is believed to have a wide range of biological effects including anti-
79 inflammatory, antioxidant, antitumour, antibacterial, and antiviral activities, which indicate potential in
80 clinical medicine [13].

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

90 2. MATERIALS AND METHODS

91 2.1 COLLECTION OF MATERIALS AND SAMPLES:

92 2.1.1 Plant materials:

93 All the spices used in this study were purchased from the local grocery store.

Comment [D3]: Please specify the common name of the spices and the scientific name of the related plant

94 2.1.2 Bacterial cultures:

95 Gram positive bacteria *Staphylococcus aureus* and *Bacillus subtilis* and Gram negative bacteria *E. coli*,
96 *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* used in this study were collected from
97 the local hospital and IMViC tests were performed to confirm the identity of these strains. The purpose
98 for selecting the above bacteria was because all of them are potential opportunistic pathogens
99 commonly associated with bacterial infections.

Comment [D4]: How the bacteria were collected? The resource and method for collection.

Comment [D5]: Please give a reference

Comment [D6]: Please give references

100 2.2 METHONAL EXTRACTION:

101 Spice extracts were prepared as mentioned previously to obtain their active components
102 which are organic in nature. About 10 g of powdered spice was extracted with a mixture of
103 dichloromethane and methanol (1:1, v/v) (2×50 ml) under reflux for 30 min and filtered to obtain
104 isolate these extract with active organic compounds. The filtrates were combined and concentrated to
105 dryness under reduced pressure at 45°C with a rotary evaporator and were stored at 4°C until further
106 use.

Comment [D7]: Please give the reference

108 2.3 PREPARATION OF INOCULUM:

109 All the cultures obtained were stored and maintained on nutrient agar slants at 4°C until
110 further use. Before performing agar well assay, each of the cultures were inoculated onto nutrient
111 broth and incubated overnight at 37°C in order to obtain a rapidly growing culture.

Comment [D8]: Give the gradients of the medium or the producer if a commercial medium was used.

Comment [D9]: Please give the maximum time for storage under this condition.

114 2.4 ANTIMICROBIAL ASSAY BY AGAR WELL DIFFUSION:

115 Muller Hinton agar plates were prepared and wells of 7 mm in diameter were cut using a well
116 borer. In few plates the wells were filled with 50 µl of the concentrated extracts. Extracts in different
117 combinations were also prepared in the ratio of 1:1 and the wells were then added with the 50 µl of
118 the combined extract in the Mueller Hinton plates spread with different cultures and were incubated at
119 37°C for 24 hrs. To check for sensitivity of the organisms to the extracts and to find if plasmid is the
120 cause for resistance in the organisms exhibiting resistance.

Comment [D10]: Please give the reference of the medium and the size of plates

Comment [D11]: With this analysis, you can not related to the resistance to the presence of plasmid.

122 2.5 PLASMID ISOLATION BY ALKALINE LYSIS METHOD:

123 Aliquot of 1.5 mL of overnight culture was taken in a micro-centrifuge tube and centrifuged at
124 13000 rpm for 3 min at 4°C. The supernatant was discarded and pellet was resuspended in 150 µl of
125 ice-cold Alkaline Lysis Solution 1 (50mM Glucose, 25mM Tris-Cl (pH 8.0), and 10mM EDTA (pH
126 8.0)) and vortexed vigorously. Then 250 µl of Alkaline Lysis Solution 2 (10N NaOH, 10% SDS)
127 were added and mixed gently, followed by addition of 250 µl of Alkaline Lysis Solution 3 (5M
128 potassium acetate, glacial acetic acid) and centrifuged at 13000 rpm for 15 min at 4°C. Supernatant
129 containing dsDNA was collected in a fresh tube without any traces of pellet. dsDNA was precipitated
130 by adding 2x volumes of absolute ethanol and vortexed. The mixture was incubated at -20°C for 1
131 hour followed by centrifugation at 13000 rpm for 10 min at 4°C. Supernatant was discarded and the
132 pellet was rinsed with 70% ethanol and centrifuged at 13000 rpm for 5 min at 4°C. The pellet was air-
133 dried and resuspended in 50 µl of sterile DEPC water and stored at -20°C.

Comment [D12]: Use xg or give the model and producer of centrifuge. For all the centrifugation, rpm should be presented as xg

Comment [D13]: Please give the complete name

135 2.6 TRANSFORMATION:

136 The competent cells were thawed on ice and ~ 5 µl of DNA was added to 100 µl of cells and mixed
137 gently. The tubes were incubated on ice for about 30 min and subjected to heat shock at 42°C in a
138 water bath for 90-120 seconds. Tubes were replaced on ice for ~2 min and 900 µl of pre warmed LB
139 (with no antibiotic added) was added and then incubated at 37°C with gentle agitation for 45 min to
140 1hr. Then 100 µl to 200 µl of this culture was spread on selective media (LB agar with vancomycin)
141 and incubated at 37°C overnight.

Comment [D14]: What are the competent cells? Where or how you got them? If they were purchased, you should give the producer.

Comment [D15]: How about the concentration?

Comment [D16]: reference

Comment [D17]: How to check or verify the transformants?

143 3. Results and Discussion

144 Here, the isolation and identification of bacterial isolates should be given.

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(Table 1): Antibiotic assay using disc diffusion method:

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.

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Table 1: Antibiotic assay using disc diffusion method

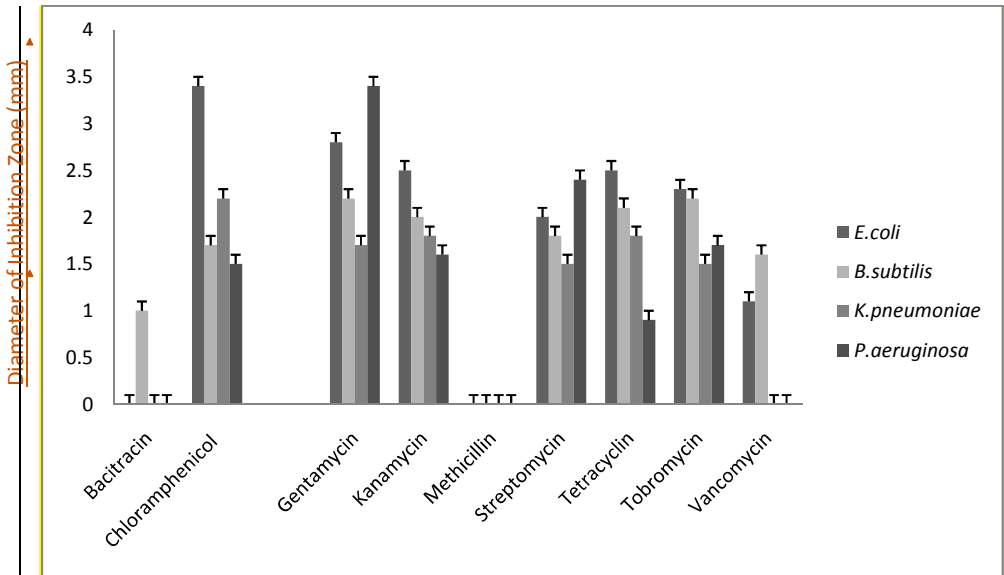
Antibiotic (mcg/disc)	<i>E. coli</i> (mm)	<i>B. subtilis</i> (mm)	<i>K. pneumoniae</i> (mm)	<i>P. aeruginosa</i> (mm)	<i>S. aureus</i> (mm)
Bacitracin (10)	-	1	-	-	2
Chloramphenicol (30)	3.4	1.7	2.2	1.5	3.2
Gentamycin (50)	2.8	2.2	1.7	3.4	2.4
Kanamycin (30)	2.5	2	1.8	1.6	2.4
Methicillin (10)	-	-	-	-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

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

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(Fig 1). Antimicrobial assay of different antibiotics on *E. coli*, *B. subtilis*, *K. pneumoniae* and *P. aeruginosa* using disc diffusion method representing the antibiotic used on X-axis and the sizes of the zones of inhibition on Y-axis (in mm).

3.1 Antimicrobial activity of spices extracted in organic solvent:

Among the selected spices cinnamon exhibited inhibition against *E. coli*, *B. subtilis* and *S. aureus* but *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* but 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 showing higher zone of inhibition up to 1 mm. 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*.



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184 {Fig 2}. Antimicrobial activity of Clove, Cinnamon, Pepper and Turmeric on (a) *E. coli*,
185 (b) *S. aureus*, (c) *B. subtilis*, (d) *K. pneumoniae* and (e) *P. aeruginosa* using organic
186 extraction method with absolute ethanol as control.

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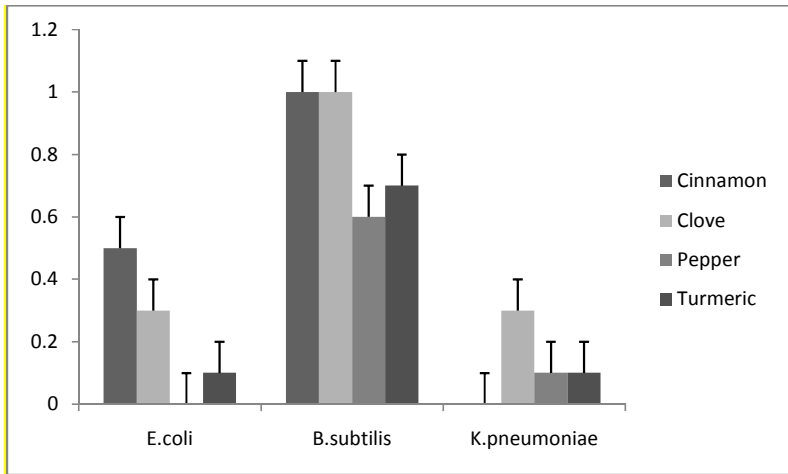
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(Table 2): Antimicrobial activity of spice extracts of Cinnamon, Clove, Pepper and Turmeric by organic extraction method on the pathogenic strains.

Spice	Inhibition zone in diameter (mm)				
	<i>E. coli</i> (mm)	<i>B. subtilis</i> (mm)	<i>K. pneumoniae</i> (mm)	<i>P. aeruginosa</i> (mm)	<i>S. aureus</i> (mm)
Cinnamon	0.5	1	-	-	0.2
Clove	0.3	1	0.3	-	0.4
Pepper	-	0.6	0.1	-	0.2
Turmeric	0.1	0.7	0.1	-	0.3

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



(Fig. 3). Antimicrobial activity of spice extracts of Cinnamon, Clove, Pepper and Turmeric using organic extraction method with absolute ethanol as control on *E. coli*, *B. subtilis* and *K. pneumoniae* representing the organisms on X-axis and zones of inhibition on Y-axis (in mm).

3.2 Antimicrobial activity of spices extracted in Phosphate Buffered Saline:-

Spices extracts such as Cinnamon, Clove, Pepper and Turmeric were checked for their antimicrobial properties on the pathogenic strains of *B. subtilis*, *E. coli*, *K. pneumoniae*, *P. aeruginosa*, *S. aureus* by isolating them in PBS (Phosphate Buffered Saline). The extracts of these spices were prepared using inorganic extraction method and then tested for antimicrobial activity on these pathogenic strains by well diffusion assay.

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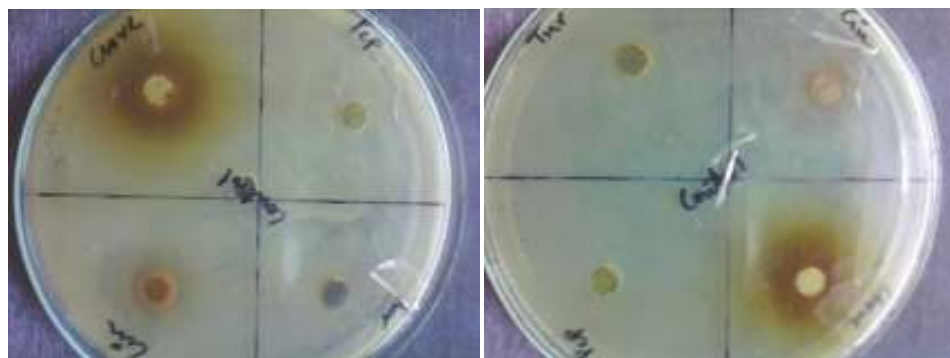
209 In *E. coli* only Cinnamon showed a considerable amount of inhibition. Whereas both
210 Cinnamon and Clove showed prominent zones of inhibition against *B. subtilis*. ~~None~~ One of the spices
211 ~~were-was~~ found to **affect** the growth in *K. pneumoniae* and *P. aeruginosa*, while Cinnamon showed
212 less **activity** compared to clove on *S. aureus*.

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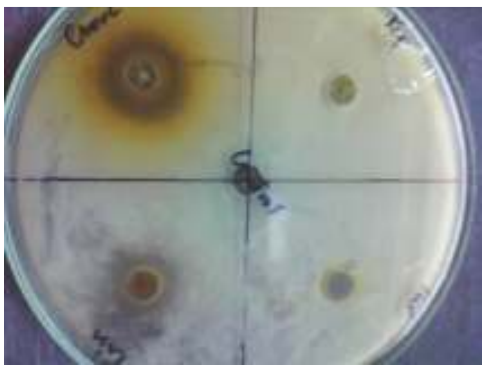
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(Fig. 4): Antimicrobial properties of Clove, Cinnamon, Pepper and Turmeric on (a) *B. subtilis*, (b) *E. coli*, (c) *K. pneumoniae*, (d) *P. aeruginosa* and (e) *S. aureus* by aqueous extraction with PBS taken as control.

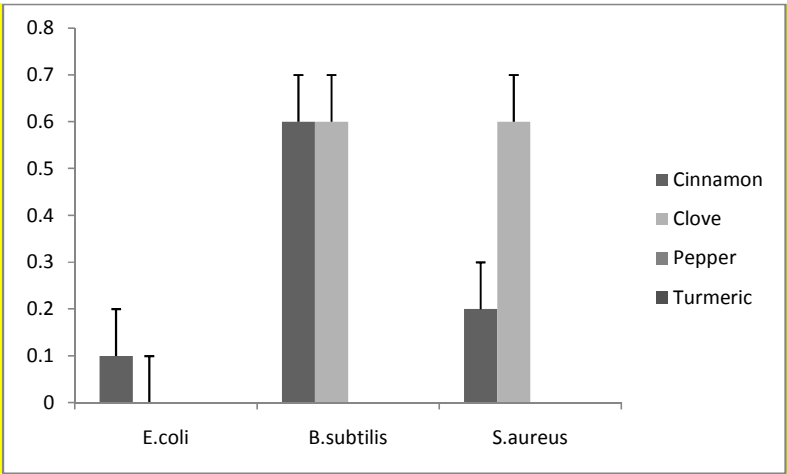
(Table 3): Antimicrobial activity of spice extracts of Cinnamon, Clove, Pepper and Turmeric prepared by using aqueous extraction (PBS) on the pathogenic strains.

Spice	Inhibition zone in diameter (mm)				
	<i>E. coli</i>	<i>B. subtilis</i>	<i>K. pneumoniae</i>	<i>P. aeruginosa</i>	<i>S. aureus</i>
	(mm)	(mm)	(mm)	(mm)	(mm)
Cinnamon	0.1	0.6	-	-	0.2
Clove	-	0.6	-	-	0.6
Pepper	-	-	-	-	-
Turmeric	-	-	-	-	-

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

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241 | **(Fig 5). Antimicrobial activity of spice extracts of Cinnamon, Clove, Pepper and**
242 **Turmeric prepared by using aqueous extraction method on *E. coli*, *B. subtilis* and *S.***
243 ***aureus* representing the organisms on X-axis and zones of inhibition on Y-axis (in**
244 **mm).**

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246 | **3.3 Antimicrobial activity by the combination of extracts in organic solvent:**

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

251 | Combination of Cinnamon + Clove, Cinnamon + Turmeric and Cinnamon + Pepper showed
252 **pronounced** zones of inhibition on *B. subtilis*. While Cinnamon+ Clove and Cinnamon+ Turmeric
253 showed inhibition but Cinnamon + Pepper did not show any inhibition on *E. coli* and none of the three
254 combinations of Cinnamon + Clove, Cinnamon + Turmeric and Cinnamon + Pepper were found to be
255 inhibiting the growth of *K. pneumoniae*, *P. aeruginosa* and *S. aureus*.

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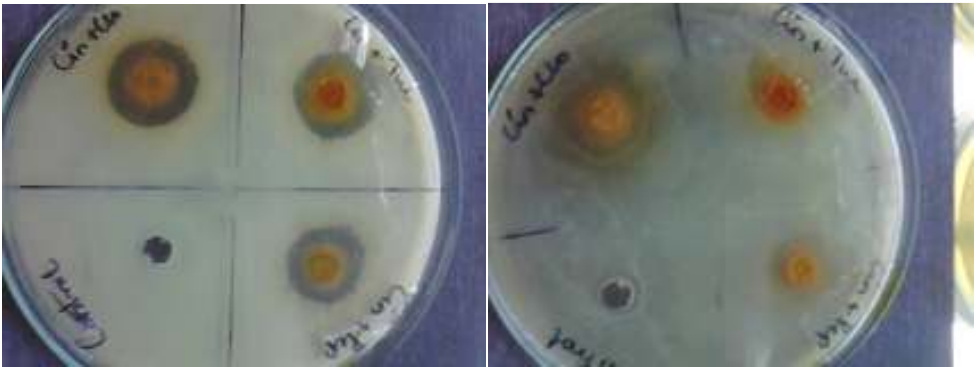
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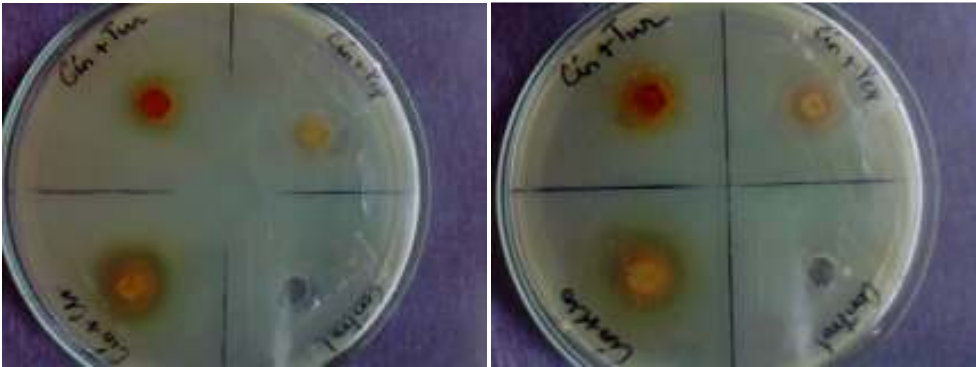
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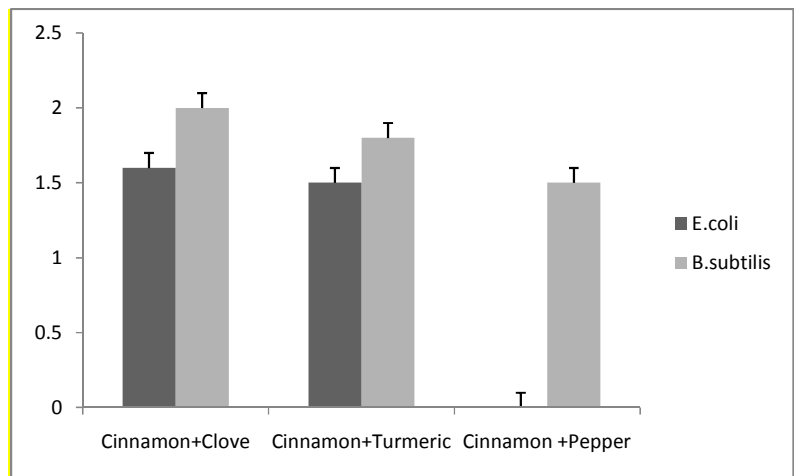
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(Fig. 6): Antimicrobial activity by the combination of spices Cinnamon + Clove, Cinnamon + Turmeric, Cinnamon + Pepper on (a) *B. subtilis*, (b) *E. coli*, (c) *S. aureus*, (d) *P. aeruginosa* and (e) *K. pneumoniae*.

(Table 4): Antimicrobial activity by the combination of extracts of Cinnamon + Clove, Cinnamon +Turmeric and Cinnamon + Pepper in organic solvent.

Combination of spices	Inhibition zone in diameter (mm)				
	<i>E. coli</i> (mm)	<i>B. subtilis</i> (mm)	<i>K. pneumoniae</i> (mm)	<i>P. aeruginosa</i> (mm)	<i>S. aureus</i> (mm)
Cinnamon+Clove	1.6	2	-	-	-
Cinnamon+Turmeric	1.5	1.8	-	-	-
Cinnamon + Pepper	-	1.5	-	-	-

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



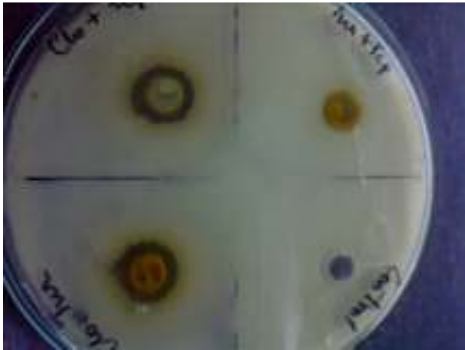
(Fig 7). Antimicrobial activity by the combination of extracts of Cinnamon + Clove, Cinnamon +Turmeric and Cinnamon + Pepper on *E. coli* and *B. subtilis* representing the combinations on X-axis and the zones of inhibition on Y-axis.

3.4 Antimicrobial activity by the combination of extracts:

Combination of Clove+ Turmeric, Clove + Pepper, Turmeric +Pepper were also checked for their inhibitory activity on pathogenic strains of *B. subtilis*, *E. coli*, *K. pneumoniae*, *P. aeruginosa* and *S. aureus* and the results were as follows.

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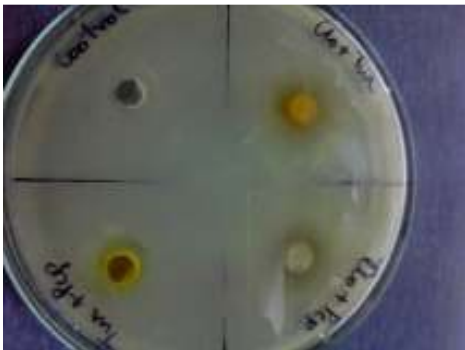
287 All the three combinations of Clove+ Turmeric, Clove + Pepper, Turmeric +Pepper were found to be
288 equally effective against *E. coli*, whereas only Clove + Turmeric and Clove +Pepper were found to be
289 inhibiting *B. subtilis*. Turmeric+Clove didn't show any inhibition. -None of the three combinations of
290 Clove+ Turmeric, Clove + Pepper, Turmeric +Pepper were found to be inhibiting the growth in *K.*
291 *pneumoniae* and *P. aeruginosa* and only combination of Turmeric+ Pepper was found to be inhibiting
292 *S. aureus*.



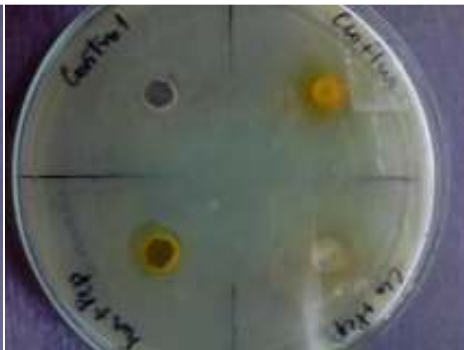
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{Fig 8}: Antimicrobial activity by the combination of spices Clove + Turmeric, Clove + Pepper, Turmeric+Pepper on (a) *B. subtilis*, (b) *E. coli*, (c) *P. aeruginosa*, (d) *S. aureus* and (e) *K. pneumoniae*.

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{Table 5}: Antimicrobial activity by the combination of spices Clove+ Turmeric, Clove + Pepper and Turmeric +Pepper.

Combination	Inhibition zone in diameter (mm)				
	<i>E. coli</i> (mm)	<i>B. subtilis</i> (mm)	<i>K. pneumoniae</i> (mm)	<i>P. aeruginosa</i> (mm)	<i>S. aureus</i> (mm)
Clove+ Turmeric	1.5	1.2	*	*	*
Clove+ Pepper	1	1.2	*	*	*
Turmeric+ Pepper	1.5	*	*	*	1.2

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

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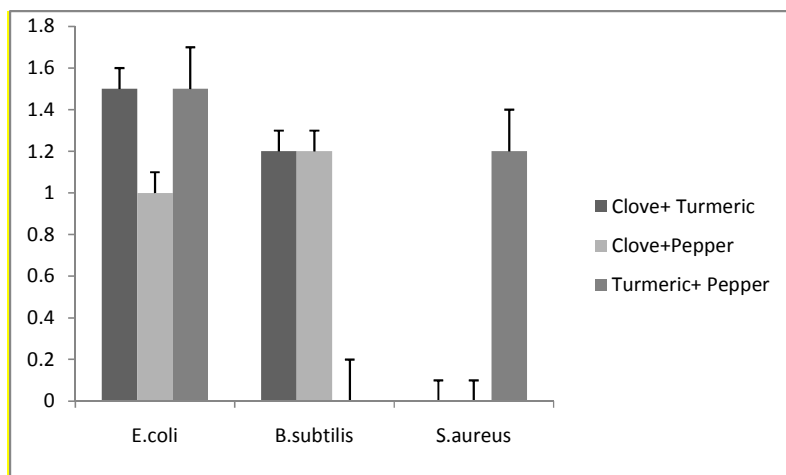
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(Fig 9). Antimicrobial activity by the combination of spices Clove + Turmeric, Clove + Pepper and Turmeric + Pepper on *E. coli*, *B. subtilis* and *S. aureus* representing the organisms on X-axis and zones of inhibition on Y-axis.

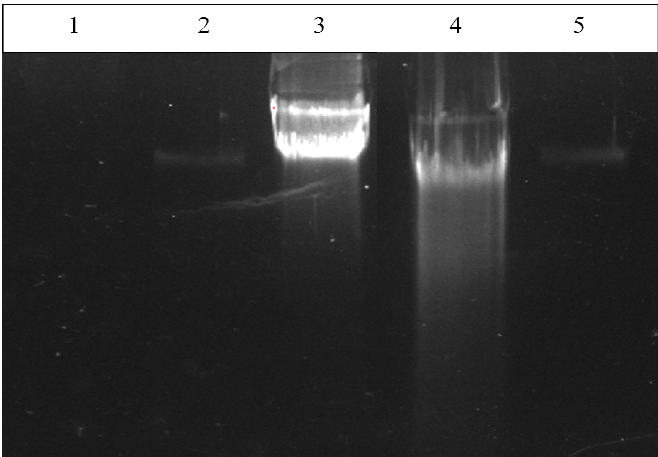
3.5 Plasmid isolation:

Plasmid isolation was performed using alkaline hydrolysis method and the plasmids were isolated from each culture and run on 1% agarose gel and checked under a gel documentation system (Bio-rad) and 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 1.75 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 which shows high copy number. Hence high copy number of resistance plasmid present in a bacterial cell showed higher resistance of the bacteria [15].

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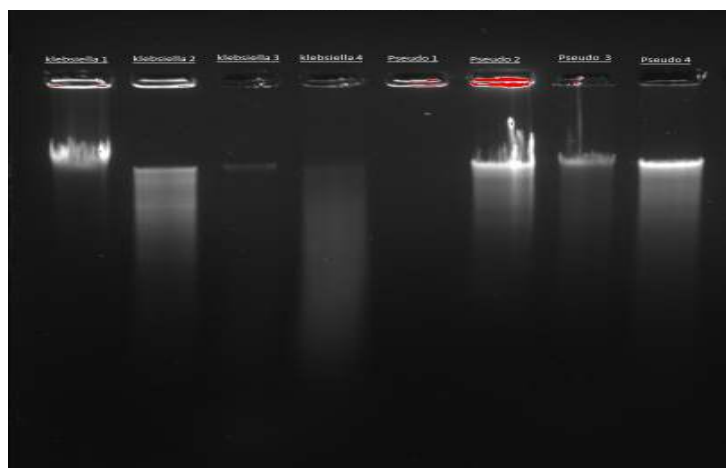
(Fig_10): Plasmid isolated from the 5 test organisms *E. coli* (1), *B. subtilis* (2), *K. pneumoniae* (3), *P. aeruginosa* (4) and *S. aureus* (5).

3.6 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 media factor. The transformed colonies were selected randomly and were grown in flasks for overnight. Their plasmids were isolated once again by alkaline lysis and were checked for the presence of the transformed plasmid. 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.

Comment [D22]: These are method

Comment [D23]: How about the transformation efficiency?



*The number indicates the colony number on the transformed plate

(Fig. 11): Plasmids isolated from transformed colonies of *E. coli* DH5- α transformed using plasmids obtained from *K. pneumoniae* and *P. aeruginosa*.

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* DH5 α which was initially sensitive to Cinnamon became resistant to it, whereas it was 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 may be 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 5): Antimicrobial activity of spices on the transformed colonies of *E. coli* Dh5α where transformation was performed using plasmid isolated from *P. aeruginosa* and *K. pneumoniae*.

Spice	Inhibition zone in diameter (mm) of transformant				
▲	Control <i>E. coli</i> DH5α (mm)	Transformed Colony 1 using <i>P.</i> <i>aeruginosa</i> plasmid (mm)	Transformed Colony 2 using <i>P.</i> <i>aeruginosa</i> Plasmid (mm)	Transformed Colony 2 using <i>K.</i> <i>pneumoniae</i> plasmid (mm)	Transformed Colony 3 Using <i>K.</i> <i>pneumoniae</i> plasmid (mm)
Cinnamon	1.3	1.5	-	-	1.5
Clove	-	1.4	2.1	1.5	1.7
Pepper	-	-	-	-	-
Turmeric	1	1.2	1.6	1.1	1

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

**E. coli* DH5α was used as control.

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 flavour [16]. Spices are considered as rich source of bio-active antimicrobial compounds [17]. Numerous studies have been published on the antimicrobial activities of plant extracts against different types of microbes, including food borne pathogens [18,19,20]. It has been reported that spices owe their antimicrobial properties mostly to the presence of alkaloids, phenols, glycosides, steroids, essential oils, coumarins and tannins [21]. 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 [22]. 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 [23]. 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 [24]. Many studies claim that phenolic substances present in spices may be responsible for antimicrobial activity [25]. Antibacterial activity of selected dietary spice was closely related to the concentration of phenolic compounds [26]. In this study, methanol and dichloromethane extracts of Cinnamon, Clove, Pepper

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401 and Turmeric proved to be more effective than the aqueous extracts of Cinnamon, Clove, Pepper and
402 Turmeric.

403 Though many different herbs and spices were tested for their antimicrobial properties, fewer
404 investigations have been carried out to study the synergistic effect of such substances. Our study
405 indicated that spices showed antimicrobial properties but even greater antimicrobial activity was
406 expressed by the combining the spices [27] because when cinnamon and clove were used
407 individually they showed zones of inhibition in the range of 0.2 to 1mm as compared to combination of
408 spices which showed increase in zones of inhibition from 1.5 to 2 mm in diameter. In general, Gram
409 negative bacteria possessing plasmid showed greater resistance to the spices when used individually
410 and in combined form [28]. Strongest antimicrobial effects were shown by cinnamon and cloves
411 against *S. taphylococcus aureus*, *K. lebsiella pneumoniae*, *E. scherichia coli*, *Enterococcus faecalis*,
412 *Mycobacterium smegmatis*, *Micrococcus luteus*, and *Candida albicans* used as test strains, with
413 inhibition zones between <10 and >30 mm by the disc-diffusion method [29]. Cinnamic aldehyde and
414 eugenol extracted from cinnamon and clove at 0.03% and 0.04% concentrations showed antibacterial
415 properties against *S. taphylococcus aureus*. *Bacillus* sp. strains were inhibited by cinnamic aldehyde
416 and eugenol extracted from cinnamon and clove at just 0.1–1.0% w/v, 0.06% v/v concentration
417 [30]. Gram positive bacteria are more susceptible towards the pepper extracts than gram negative
418 bacteria. However, there are reports of occurrence of antibacterial activity against *S. aureus*, *E. coli*,
419 *B. megaterium*, *B. sphaericus*, *B. polymyxa* [31,32]. Spices like turmeric showed maximum activity
420 against gram positive than compared to gram negative bacteria individually and by mixtures [33].
421 *Pseudomonas*, specifically *P. aeruginosa* are the least sensitive group of bacteria to the action of
422 essential oils and bioactive components of plant-origin [34].

423 *K. pneumoniae* organisms are often resistant to multiple antibiotics. Current evidence
424 implicates plasmid as the source of the resistant genes.[35]. *K. pneumoniae* with the ability to produce
425 extended-spectrum beta-lactamases ESBL is resistant to many classes of antibiotics. The most
426 frequent antibiotic resistances include resistance to aminoglycosides, fluoroquinolones, tetracyclines,
427 chloramphenicol, and trimethoprim/sulfamethoxazole. *P. aeruginosa* is an opportunistic, nosocomial
428 pathogen of immunocompromised individuals. *P. aeruginosa* is naturally resistant to a large range of
429 antibiotics. *P. aeruginosa* easily acquires resistance either by mutation in chromosomally encoded
430 genes or by the horizontal gene transfer of antibiotic resistance determinants. Development of
431 multidrug resistance by *P. aeruginosa* isolates requires several different genetic events, including
432 mutations and/or horizontal transfer of antibiotic resistance genes.
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434 Resistance (R) plasmids, historically known as R-factors, contain genes that provide
435 resistance against antibiotics or toxins. In the current study bacteria containing plasmid were identified
436 by subjecting each of them to plasmid isolation and running on agarose gel to observe the plasmid. It
437 was observed that two of the cultures *K. pneumoniae* and *P. aeruginosa* showing resistance to most
438 of the antibiotics and also to the spice extracts displayed a high copy number of their plasmids. The

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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.

CONCLUSION:

Spices have antimicrobial properties which if present in diet may be beneficial especially if used in combinations. Plasmid borne resistance to spices seems to be naturally present in some bacteria such as *P. aeruginosa*, hence having a possibility of transferring the resistance to other species which might be of some significance in people who may be at risk of acquiring opportunistic infections.

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COMPETING INTERESTS:

The authors declare that they do not have any competing interests.

AUTHORS' CONTRIBUTIONS:

SB designed the work and edited the manuscript, APK performed major portion of the work and prepared the manuscript, KR performed a part of the experiments, MSR, KR and AN helped with writing the manuscript writing and editing it.

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