

Original Research Article

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 the nature of resistance in the bacteria exhibiting resistance.

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 pneumonia*), *P.aeruginosa*(*Pseudomonas aeruginosa*) and *S.aureus*(*Staphylococcus aureus*).

Place and duration of study: Department of Microbiology, Osmania University, during the year 2013.

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 bacterial strains.

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:

Bacteria are ubiquitous and continuously adapting to newer and harsher environments. Even though antibiotics are effective in bacterial killing, microbes are continuously developing resistance to most

Comment [J1]: Space pls

Comment [J2]: Is it plasmid borne resistance or chromosomal resistances

Comment [J3]: Space

Comment [J4]: Add space before the bracket pls Also check the organisms

Comment [J5]: Space, which country? Was the research carried out throughout the year?

Comment [J6]: Space

Comment [J7]: Pls space the organisms name

Comment [J8]: bacteria

Comment [J9]: rephrase

Comment [J10]: microbes are

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 and tougher strains are being identified every year [3]. For instance, 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. Mycobacteria, the genus harbouring 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 [4,5]. Multiple drug resistance in Mycobacteria is prevalent worldwide and billions of dollars are spent every year to devise effective treatment strategies against the pathogen. 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 [6].

Spices are essential components of Indian cuisines since ancient times. These are being used in minute amounts to impart flavour, taste and aroma in food preparation [7]. Many spices have potent antimicrobial properties [8]. 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 a local antiseptic. The oil also contains about 10% acetylene and small quantities of gallic acid, sesquiterpenes, furfural, vanillin, and methyl-n-amyl ketone. Essential oils contain very complex mixtures of compounds, varied from monoterpenes to sesquiterpenes [9]. Black Pepper used globally as a spice and seasoning and 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-3 α ,8 α -dimethyl-5 α -(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,

Comment [IJ11]: space

Comment [IJ12]: delete

Comment [IJ13]: space

Comment [IJ14]: is MRSA, *E. coli*? I believe the sentence is continues? What about *E. coli* 0157:H7, other serotypes of *E. coli*?

Comment [IJ15]: Please take your time and check those words that need to be spaced

Comment [IJ16]: coma

Comment [IJ17]: Space. Your introduction is scanty with reference to your topic.

Comment [IJ18]: space, pls, discuss extensively on the clinical significance of plasmid born bacteria,

Comment [IJ19]: there is no need for paragraph, also check the spacing

Comment [IJ20]: check the space, the

Comment [IJ21]: local antiseptic

Comment [IJ22]: check your copy and past materials

Comment [IJ23]: Rephrase

Comment [IJ24]: space

Comment [IJ25]: the

Comment [IJ26]: p

Comment [IJ27]: t

Comment [IJ28]: t

67 antioxidant, antitumour, antibacterial, and antiviral activities, which indicate potential in clinical medicine
68 [10].

Comment [IJ29]: Please your flow is ok, your topic is not explicitly discussed in your introduction. Please I advise you recast.

69 The present study was planned to check the antimicrobial activity of these spices on some commonly
70 found normal flora which are frequently opportunistic pathogens. The purpose of the study was to check
71 whether these spices have any antimicrobial activity to contribute to general well being.

Comment [IJ30]: This is your study statement

72
73
74

75 2. MATERIALS AND METHODS

76 2.1 COLLECTION OF MATERIALS AND SAMPLES:

77 2.1.1 Plant materials:

78 All the spices used in the experiment were purchased from the local grocery store.

Comment [IJ31]: This study

79 2.1.2 Bacterial cultures:

Comment [IJ32]: space

80 Gram positive bacteria *S. aureus* and *B. subtilis* and Gram negative bacteria *E. coli*, *P. aeruginosa* and *K. pneumoniae* used in the experiment were collected from the local hospital and IMViC tests were
81 performed to isolate to isolate pure cultures of these strains. The purpose for selecting the above bacteria
82 was because all of them are potential opportunistic pathogens commonly associated with bacterial
83 infection.

Comment [IJ33]: space

Comment [IJ34]: this study

Comment [IJ35]: repetition

Comment [IJ36]: bacteria infections

85 2.2 METHONAL EXTRACTION:

86 About 10 g of powdered drug was extracted with a mixture of dichloromethane and methanol (1:1,
87 v/v) (2×50 ml) under reflux for 30 min and filtered. The filtrates were combined and concentrated to
88 dryness under reduced pressure at 45°C with a rotary evaporator and were stored at 4°C until further use.

Comment [IJ37]: close space

Comment [IJ38]: which powered drug?

Comment [IJ39]: Why?

90 2.3 PREPARATION OF INOCULUM:

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

Comment [IJ40]: At what temperature

95 2.4 ANTIMICROBIAL ASSAY BY AGAR WELL DIFFUSION:

96 Muller Hinton agar plates were prepared and wells of 7mm were cut. In few plates the wells were
97 filled with 50µl of the concentrated extract. Extracts in different combinations are prepared in 1:1 ratio and

Comment [IJ41]: Using ?

Comment [IJ42]: extracts

Comment [IJ43]: were

Comment [IJ44]: ratio 1:1

the wells are then added with 50µl of the combined extract in other plates and were incubated at 37°C for 24hrs.

Comment [IJ45]: were

Comment [IJ46]: with the

Comment [IJ47]: which plate?

Comment [IJ48]: C

Comment [IJ49]: What are your basis for selection? If the clinical isolates are sensitive why testing for plasmids

Comment [IJ50]: space

Comment [IJ51]: space

Comment [IJ52]: space

Comment [IJ53]: small letters

Comment [IJ54]: space

Comment [IJ55]: space

Comment [IJ56]: .

Comment [IJ57]: Close space

2.5 PLASMID ISOLATION BY ALKALINE LYSIS METHOD:

1.5 mL of overnight culture was taken in an Eppendorf tube and centrifuged at 13000 rpm for 3min at 4°C. The supernatant was discarded and pellet was resuspended in 150µl of ice-cold Alkaline Lysis Solution 1 (50mM Glucose, 25mM TrisCl (pH 8.0), 10mM EDTA (pH 8.0)) and vortexed vigorously. 250µl of Alkaline Lysis Solution 2 (10N NaOH, 10% SDS) was added and mixed gently, followed by addition of 250µl of Alkaline Lysis Solution 3 (5M Potassium Acetate, Glacial Acetic Acid) and centrifuged at 13000 rpm for 15min at 4°C. Supernatant containing dsDNA was collected in a fresh tube without any traces of pellet. dsDNA was precipitated by adding 2x volumes of absolute ethanol and vortexed. The mixture was incubated at -20°C for 1 hour followed by centrifugation at 13000 rpm for 10min at 4°C. Supernatant was discarded and the pellet was rinsed with 70% ethanol and centrifuged at 13000 rpm for 5min at 4°C. The pellet was air-dried and resuspended in 50µl of sterile DEPC water and stored at -20°C.

2.6 TRANSFORMATION:

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

Comment [IJ58]: space

3. Results and Discussion:

Comment [IJ59]: Space

(Table 1): Antibiotic assay using disc diffusion:

Comment [IJ60]: Disc diffusion method

Antibiotic assay was performed on each of the cultures using antibiotic discs containing antibiotic at the concentrations of 10, 30 and 50µg/disc.

Comment [IJ61]: What prompted the choice of the antibiotics selected?

Comment [IJ62]: space

Antibiotic (mcg/disc)	<i>E.coli</i>	<i>B.subtilis</i>	<i>K.pneumoniae</i>	<i>P.aeruginosa</i>	<i>S.aureus</i>
Bacitracin (10)	-ve	+ve	-ve	-ve	+ve
Chloramphenicol (30)	+ve	+ve	+ve	+ve	+ve
Gentamycin (50)	+ve	+ve	+ve	+ve	+ve

Kanamycin (30)	+ve	+ve	+ve	+ve	+ve
Methicillin (10)	-ve	-ve	-ve	-ve	+ve
Streptomycin(10)	+ve	+ve	+ve	+ve	+ve
Tetracyclin(30)	+ve	+ve	+ve	+ve	+ve
Tobromycin (10)	+ve	+ve	+ve	+ve	+ve
Vancomycin(30)	+ve	+ve	-ve	-ve	+ve

*+ve indicates inhibition and –ve indicates no-inhibition.

3.1 Antimicrobial activity of spices extracted in organic solvent:

In *E. coli*, spice extracts of Clove, Turmeric and Cinnamon showed prominent zone of inhibition among which Clove showed higher inhibitory activity among other spices whereas, all the spices showed inhibitory activity against *S.aureus* but highest was expressed by Clove. All the spice extracts showed prominent zones of inhibition with Cinnamon and Clove showing higher zone of inhibition against *B.subtilis*. 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*.



(a)

(b)

Comment [IJ63]: what is + and -. Did you measure the zone of inhibition in mm and compare it to CLSI guideline

Comment [IJ64]: recast

Comment [IJ65]: compared to other spices. All the spices

Comment [IJ66]: Join the *E. coli* and *S. aureus* together and make a sentence.

Comment [IJ67]: What is the diameter in mm of the zone of inhibition

Comment [IJ68]: Italize pls. I will appreciate if the spices are picked one at a time and discussed. E.g among the selected spices, Clove showed significant antimicrobial activity against *E. coli*, *S. aureus*, *B. subtilis* and *K. pneumonia*, Cinnamon showed activity against while Turmeric showed



(c)

(d)



(e)

(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.

Comment [IJ69]: and

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

	<i>E.coli</i>	<i>B.subtilis</i>	<i>K.pneumoniae</i>	<i>P.aeruginosa</i>	<i>S.aureus</i>
Cinnamon	+ve	+ve	-ve	-ve	+ve
Clove	+ve	+ve	+ve	-ve	+ve
Pepper	-ve	+ve	+ve	-ve	+ve

Turmeric	+ve	+ve	+ve	-ve	+ve
----------	-----	-----	-----	-----	-----

*+ve indicates inhibition and -ve indicates no-inhibition.

Comment [I170]: what is the diameter of the zone of inhibition.

3.2 Antimicrobial activity of spices extracted in PBS:

Comment [I171]: In full pls

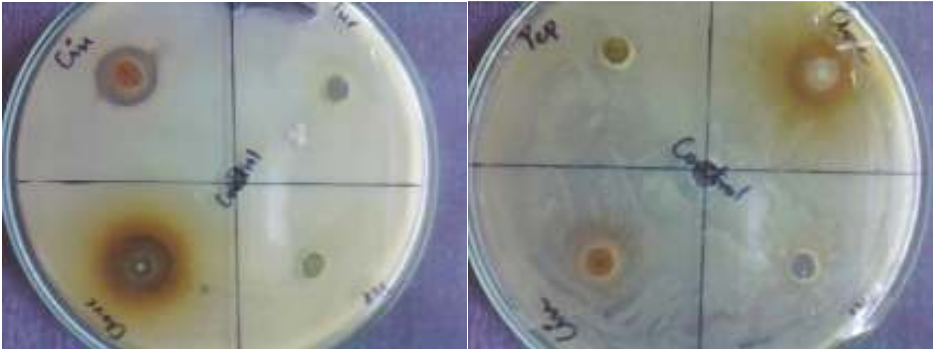
Spices extracts such as Cinnamon, Clove, Pepper and Turmeric were checked for their antimicrobial properties on the pathogenic strains of *B.subtilis*, *E.coli*, *K.pneumoniae*, *P.aeruginosa*, *S.aureus* by isolating them in PBS (phosphate buffered saline). The extracts of these spices were prepared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

Comment [I172]: remove

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 effect the growth in *K.pneumoniae* and *P.aeruginosa*, while Cinnamon showed less inhibition compared to Clove on *S.aureus*.

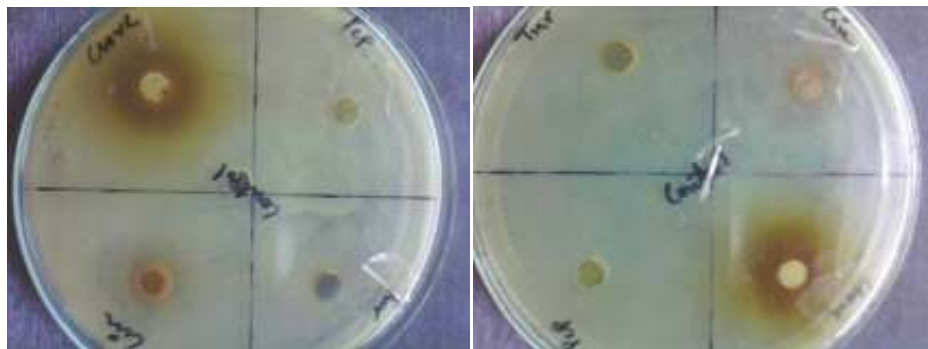
Comment [I173]: affect

Comment [I174]: activity



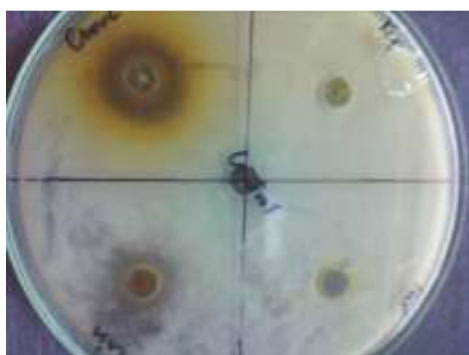
(a)

(b)



(c)

(d)



(e)

(Fig 2):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.

	<i>E.coli</i>	<i>B.subtilis</i>	<i>K.pneumoniae</i>	<i>P.aeruginosa</i>	<i>S.aureus</i>
Cinnamon	+ve	+ve	-ve	-ve	+ve
Clove	-ve	+ve	-ve	-ve	+ve
Pepper	-ve	-ve	-ve	-ve	-ve
Turmeric	-ve	-ve	-ve	-ve	-ve

*+ve indicates inhibition and –ve indicates no-inhibition.

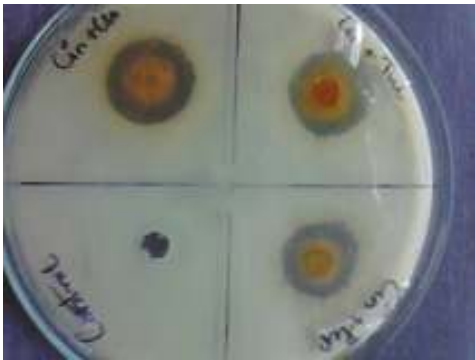
3.3 Antimicrobial activity by the combination of extracts in organic solvent:

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* and the results were as follows.

Combination of Cinnamon + Clove, Cinnamon + Turmeric and Cinnamon + Pepper showed very prominent 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*.

Comment [1775]: ,

Comment [1776]: Pronounced/significant



(a)



(b)



(c)



(d)



(e)

(Fig 3): Antimicrobial activity by the combination of spices Cinnamon + Clove, Cinnamon + Turmeric, Cinnamon + Pepper on (a) *B.subtilis*, (b)*E.coli*, (c)*P.aeruginosa*, (d)*S.aureus* and (e)*K.pneumoniae*.

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

Combination	<i>E.coli</i>	<i>B.subtilis</i>	<i>K.pneumoniae</i>	<i>P.aeruginosa</i>	<i>S.aureus</i>
Cinnamon+Clove	+ve	+ve	-ve	-ve	-ve
Cinnamon+Turmeric	+ve	+ve	-ve	-ve	-ve
Cinnamon +Pepper	-ve	+ve	-ve	-ve	-ve

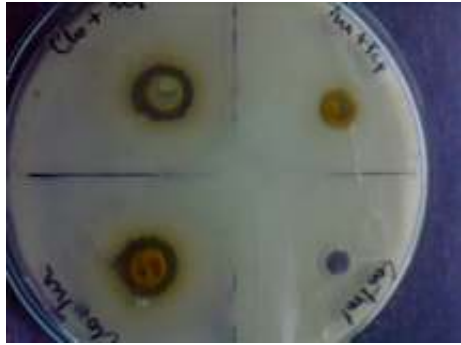
*+ve indicates inhibition and –ve indicates no-inhibition.

3.4 Antimicrobial activity by the combination of extracts:

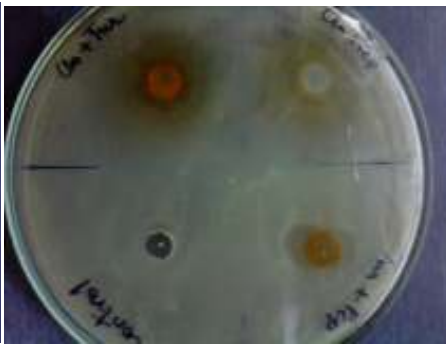
Combination of Clove+ Turmeric, Clove + Pepper, Turmeric +Pepper were also checked for their inhibitory activity on pathogenic strains of *B.subtilis*, *E.coli*, *K.pneumoniae*, *P.aeruginosa*and*S.aureus*and the results were as follows.

All the three combinations ofClove+ 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* but Turmeric+Clove didn't show any inhibition. None of three combinations ofClove+ 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*.

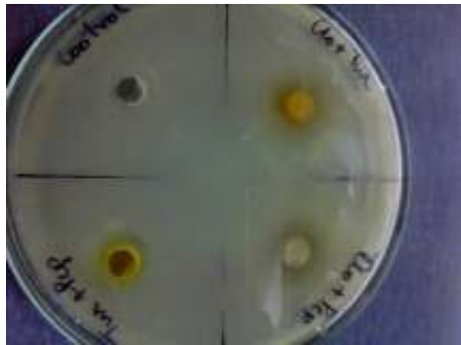
Comment [177]: the



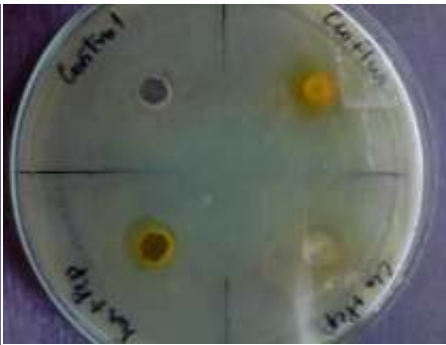
(a)



(b)



(c)



(d)



(e)

(Fig 4): 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*.

211 (Table 5): Antimicrobial activity by the combination of spices Clove+ Turmeric, Clove +
212 Pepper and Turmeric + Pepper.

Combination	<i>E.coli</i>	<i>B.subtilis</i>	<i>K.pneumoniae</i>	<i>P.aeruginosa</i>	<i>S.aureus</i>
Clove+ Turmeric	+ve	+ve	-ve	-ve	-ve
Clove+Pepper	+ve	+ve	-ve	-ve	-ve
Turmeric+ Pepper	+ve	-ve	-ve	-ve	+ve

213 *+ve indicates inhibition and –ve indicates no inhibition.

Comment [IJ78]: pls compare your result with other peoples findings and discuss it extensively.

214 3.5 Plasmid isolation:

215 Plasmid isolation was performed using alkaline hydrolysis method and the plasmids were isolated from
216 each culture and run on 1% agarose gel and checked under an Gel Documentation system (Bio-rad) and
217 it was observed that the *E.coli* culture used did not contain any plasmid whereas *B.subtilis* and *S.aureus*
218 had plasmids with a small copy number but *K.pneumoniae* and *P.aeruginosa* had a moderate copy
219 number of their plasmid.

Comment [IJ79]: a

Comment [IJ80]: a gel documentation system

Comment [IJ81]: space

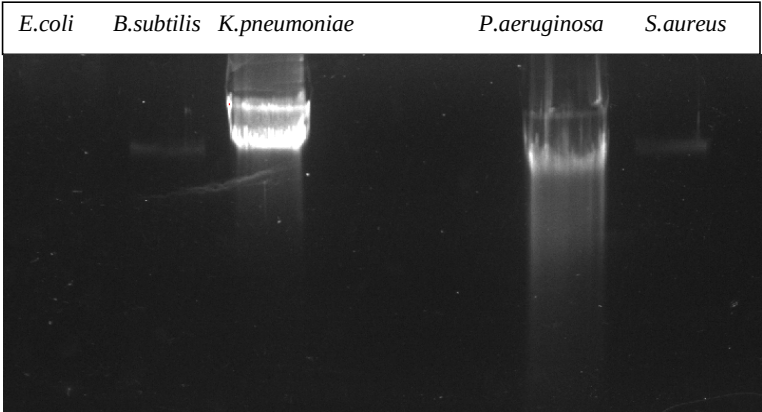
Comment [IJ82]: How did you get the copy number

220 (Fig5): Plasmid isolated from the 5 test organisms *E.coli*, *B.subtilis*, *K.pneumoniae*,
221 *P.aeruginosa* and *S.aureus*.

Comment [IJ83]: let the font size be the same pls. why the choice of the selected isolates. Where they resistant?, when you compared the standard antibiotics with the spice are there significant difference between the two?

222

223



Comment [IJ84]: Please use picture management or paint to number your pictures and label the wells.

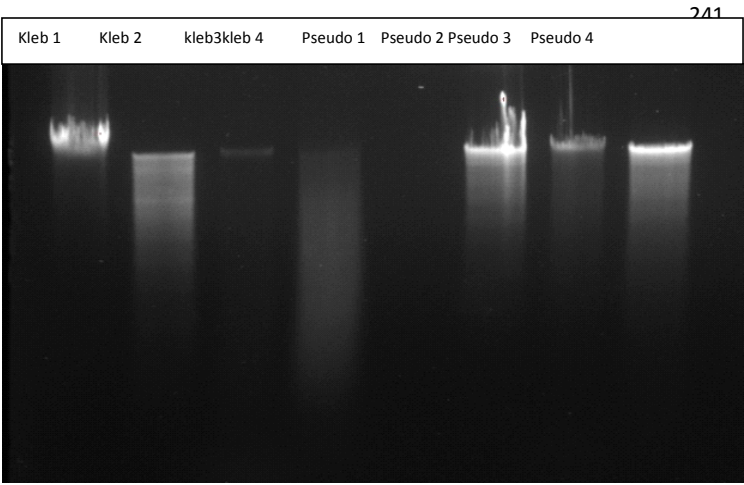
230 **K.pneumoniae*-Klebsiella pneumonia, *P.aeruginosa* – Pseudomonas aeruginosa.

231 3.6 Transformation:

232 Transformation experiment was performed by using the plasmids isolated by alkaline lysis method and
233 the transformed culture was allowed to grow on media containing the antibiotic vancomycin as the
234 selective media and the transformed colonies were selected randomly and were grown in flasks and their
235 plasmids were isolated once again by alkaline lysis and were checked for the presence of the
236 transformed plasmid. It was found that colonies 2 and 3 of *K.pneumoniae* and colonies 2,3 and 4 of
237 *P.aeruginosa* were successfully transformed.

238 (Fig 6): Plasmids isolated from transformed colonies of *E.coli* DH5-α transformed using
239 plasmids obtained from *K.pneumoniae* and *P.aeruginosa*.

240



249 *Kleb indicates *K. pneumoniae* and Pseudo indicates *P. aeruginosa*.

250 *The number indicates the colony number on the transformed plate

251 **3.8 Antimicrobial activity of spices on the transformed colonies:**

252 Antimicrobial activity of the spices were checked using well diffusion assay method and it was
253 found to that *E.coli* Dh5α which was initially sensitive to Cinnamon became resistant to it. Whereas it was
254 resistant to Clove before transformation but became sensitive to it after transformation. Resistance to
255 Pepper and Turmeric remained the same before and after transformation. showed prominent zones of
256 inhibition on the control culture. Whereas cultures transformed using *P.aeruginosa* plasmids were
257 inhibited by Cinnamon, Clove and Turmeric. While cultures transformed using *K.pneumoniae* plasmid
258 were inhibited by only Clove and Turmeric.

Comment [IJ85]: delete

Comment [IJ86]: what is the MIC of vancomycin used to the selected isolates?

Comment [IJ87]: ?

Comment [IJ88]: Move under the picture

Comment [IJ89]: As above

Comment [IJ90]: . It

Comment [IJ91]: delete

Comment [IJ92]: why?

Comment [IJ93]: ?

Comment [IJ94]: Why? What are the health importance of this study?

259

260

261

262

263

264

265

(Table5): Antimicrobial activity of spices on the transformed colonies of *E.coli* Dh5 α where transformation was performed using plasmid isolated from *P.aeruginosa* and *K.pneumoniae*.

	Control <i>E.coli</i> Dh5 α	Transformed colony1 using <i>P.aeruginosa</i> plasmid	Transformed colony 2 using <i>P.aeruginosa</i> plasmid	Transformed colony 2 using <i>K.pneumoniae</i> plasmid	Transformed colony 3 Using <i>K.pneumoniae</i> plasmid
Cinnamon	+ve	+ve	-ve	-ve	+ve
Clove	-ve	+ve	+ve	+ve	+ve
Pepper	-ve	-ve	-ve	-ve	-ve
Turmeric	+ve	+ve	+ve	+ve	+ve

266

*+ve indicatesinhibition and –ve indicates no-inhibition.

267

**E.coli* DH5 α was used as control.

268

Discussion:

269

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 and to enhance aroma and flavour [11]. Numerous studies have been published on the antimicrobial activities of plant extracts against different types of microbes, including foodborne pathogens [12,13,14]. It has been reported that spices owe their antimicrobial properties mostly to the presence of alkaloids, phenols, glycosides, steroids, essential oils, coumarins and tannins [15]. 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 [16].

277

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 usingvarious solvents, and most of the components with antimicrobial properties are aromatic or

Comment [IJ95]: Close space

Comment [IJ96]: If discussion is separate, remove all the explanation under the tables and include it in the discussion.

Comment [IJ97]: delete and put coma

Comment [IJ98]: space

282 saturated organic compounds which are generally more soluble in solvents such as ethanol or methanol
283 [17]. In a recent study on antimicrobial activity of Australian herbs, water, ethanol and hexane were used
284 as the solvents for extraction, and it was observed that the aqueous extracts displayed little or no
285 antimicrobial activity [18]. Many studies claim that phenolic substances present in spices may be
286 responsible for antimicrobial activity [19]. Antibacterial activity of selected dietary spice was closely
287 related to the concentration of phenolic compounds [20]. In this study, methanol and dichloromethane
288 extracts of Cinnamon, Clove, Pepper and Turmeric proved to be more effective than the aqueous extracts
289 of the same.

Comment [IJ99]: ,

Comment [IJ100]: What?

291 The term synergy implies that combination of compounds to create more profound microbial action
292 may also be an important factor in using spices to kill bacteria. Though many different herbs and spices
293 were tested for their antimicrobial properties, fewer investigations have been carried out to study the
294 synergistic effect of such substances. Our study indicated that spices showed antimicrobial properties but
295 greater antimicrobial activity was expressed by the combination of the spices. [21]. In general Gram
296 negative bacteria possessing plasmid showed greater resistance to the spices used individually and
297 those used in combination [22].

Comment [IJ101]: Not necessary

Comment [IJ102]: Delete. Compare your result pls.

Comment [IJ103]: Delete. And in combined form.

298 .
299 *K.pneumoniae* organisms are often resistant to multiple antibiotics. Current evidence implicates a
300 plasmid as the source of the resistant genes. *K.pneumoniae* with the ability to produce extended-spectrum
301 beta-lactamases ESBL are resistant to many classes of antibiotics. The most frequent resistances include
302 resistance to aminoglycosides, fluoroquinolones, tetracyclines, chloramphenicol, and
303 trimethoprim/sulfamethoxazole. *P. aeruginosa* is an opportunistic, nosocomial pathogen of immune
304 compromised individuals. *P. aeruginosa* is naturally resistant to a large range of antibiotics. *P. aeruginosa*
305 easily develops acquired resistance either by mutation in chromosomally encoded genes or by the
306 horizontal gene transfer of antibiotic resistance determinants. Development of multidrug resistance by *P.*
307 *aeruginosa* isolates requires several different genetic events, including acquisition of different mutations
308 and/or horizontal transfer of antibiotic resistance genes.

Comment [IJ104]: Why?

Comment [IJ105]: is

Comment [IJ106]: of what?

Comment [IJ107]: Immunocompromised

Comment [IJ108]: .

Comment [IJ109]: .

Comment [IJ110]: /

Comment [IJ111]: You don't acquire mutation, you develop it

310 Resistance (R) plasmids, historically known as R-factors, contain genes that provide resistance
311 against antibiotics or toxins. In the current study bacteria containing plasmid were identified by subjecting
312 each of them to plasmid isolation and running on Agarose gel to observe the plasmid. It was observed
313 that two of the cultures *K.pneumoniae* and *P.aeruginosa* showing resistance to most of the antibiotics and
314 also to the spice extracts displayed a high copy number of their plasmids. The isolated plasmid was then
315 transformed into *E.coli* Dh5 α to check if the presence of the plasmid was responsible for their resistance
316 to the extracts. Transformed colonies were picked based upon their growth in the presence of the
317 antibiotic Vancomycin because in the initial study it was observed that *E.coli* Dh5 α was sensitive to

Comment [IJ112]: .

Comment [IJ113]: How?

Comment [IJ114]: on

Comment [IJ115]: v

318 Vancomycin where as *K.pneumoniae* and *P.aeruginosa* were found to be resistant so Vancomycin was
319 used in the selection of the transformed colonies.

Comment [IJ116]: Methodology repeated. Pls. delete. Please discuss your result and correlate your findings with other works done by other researcher.

320 321 **CONCLUSION:**

322
323 Hence we conclude that spice extracts can be used as effective antimicrobial agents and resistance to
324 not only the antibiotics but also to antimicrobial agents such as extracts of spices is also carried by
325 plasmid.

Comment [IJ117]: Please just suggest

Comment [IJ118]: Rephrase.

327 328 **COMPETING INTERESTS:**

329
330 The authors declare that they do not have any competing interests.

337 **References:**

- 338 1.Cohen, M.L. 2002. Changing patterns of infectious disease.Nature 406: 762-767.
339
340 2. Walsh, C. 2000. Molecular mechanisms that confer antibacterial drug resistance. Nature 406:775-
341 781.
342
343 3.Akram M, Shahid M, Khan AU: Etiology and Antibiotics Resistance Pattern of Community Acquired
344 Urinary Infections in J N M C Hospital Aligarh India. Ann ClinMicrobiolAntimicrob 2007, 6:4.
- 345 4.Dye C (2000) Tuberculosis 2000-2010: control, but not elimination. Int J TubercLung Dis 4: S146-152.
- 346 5.LoBue P (2009) Extensively drug-resistant tuberculosis. CurrOpin Infect Dis 22: 167-173..)
- 347 6. Odugbemi T: Medicinal plants as antimicrobials In: Outline and pictures of Medicinal plants from
348 Nigeria. University of Lagos press,2006: pp. 53-64.
- 349 7.Rahman, M. andGul. S. 2002. Antibacterial activity of hydrodistilled essential oil of
350 PsmmogetoncanescensN. O. Umbelliferae. Biotechnology 1: 55-60.
- 351 8.HARGREAVES, L.L., JARVIS,B.,RAWLINSON,A.P.&WOOD,J.M. 1975. The antimicrobial effects of
352 spices, herbs and extracts from these and other food plants. Scientific and Technical Surveys, British
353 Food Manufacturing Industries Research Association 88: 1–56.
- 354 9.Simon et al., 1984; Bown, 1995; EMEA, 1998; Svoboda and Hampson, 1999, Oumzil et al., 2002,
355 Esiyok et al., 2004; Gulluce et al., 2004, Li and Jiang, 2004)
- 356 10.Luthra, P.M., R. Singh and R. Chandra. 2001. Therapeutic uses of Curcuma longa (Turmeric). Indian
357 J. Clin.Biochem., 16: 153-160.

- 358 11. E. L. Souza, T. L. M. Stamford, E. O. Lima, V. N. Trajano and J. B. Filho (2005). Antimicrobial
359 effectiveness of spices: an approach for use in food conservation systems. *Braz. Arch. Biol. Technol.*
360 Vol.48 549-558. N.Uraih (2004). *Food Microbiology*. Bobpeco Publishers, Benin City, Nigeria, pp. 92-130.
361
- 362 12. Beuchat, L.R.: Antimicrobial properties of spices and their essential oils, in *Natural Antimicrobial*
363 *Systems and Food Preservation*. Eds. Y. M. Dillon and R. G. Board, CAB International, Oxon, (1994) pp.
364 167-179.,
- 365 13. Smith-Palmer, A., J. Stewart and L. Fyfe: Antimicrobial properties of plant essential oils and essences
366 against five important food-borne pathogens. *Lett. Appl. Microbiol.* **26** (1998) 118-122.,
- 367 14. Hara-Kudo, Y., A. Kobayashi, Y. Sugita-Konishi and K. Kondo: Antibacterial activity of plants used in
368 cooking for aroma and taste. *J. Food Protect.* **67** (2004) 2820-2824.).
- 369 15. Ebana, R. U. B., B.E. Madunagu, E.D. Ekpe and I. N. Otung: Microbiological exploitation of cardiac
370 glycosides and alkaloids from *Garcinia kola*, *Borreriaocymoides*, *Kola nitida* and *Citrus aurantifolia*. *J.*
371 *App. Bacteriol.* **71** (1991) 398-401.
- 372 16. Paster, N. and L. B. Bullerman: Mould spoilage and mycotoxin formation in grains as controlled by
373 physical means. *Int. J. Food Microbiol.* **7** (1988) 257-265).
- 374 17. Cowan, M. M. (1999). Plant products as antimicrobial agents. *Clin. Microbiol. Rev.*, **12**(4), 564-582.
- 375 18. Dupont, S., Caffin, N., Bhandari, B., & Dykes, G. A. (2006). In vitro antibacterial activity of Australian
376 native herb extracts against food-related bacteria. *Food Control*, **17**(11), 929-932.
377
- 378 19. Kim, J., Marshall, M. R., & Wei, C. I. (1995). Antibacterial activity of some essential oil components
379 against five foodborne pathogens. *Journal of Agricultural and Food Chemistry*, **43**(11), 2839-2845.
380
- 381 20. Shan, B., Cai, Y.-Z., Brooks, J. D., & Corke, H. (2007). The in vitro antibacterial activity of dietary spice
382 and medicinal herb extracts. *International Journal of Food Microbiology*, **117**(1), 112-119.
383
- 384 21. Satish, K.P., Moellering, R.C. and Eliopoulos, G.M., 2005. Antimicrobial combinations. In: Lorian
385 V. (Ed). *Antibiotics in laboratory medicine*. Lippincott Williams and Wilkins, Philadelphia.
- 386 22. Gutierrez, J., Barry-Ryan, C. and Bourke, P. 2008. The antimicrobial efficacy of plant essential
387 oil combinations and interactions with food ingredients. *International Journal of Food Microbiology* **124**:
388 91-97.

389
390
391
392