

ORIGINAL ARTICLE

Bioactive Compound Rich Indian Spices Suppresses the Growth of β -lactamase Produced Multidrug Resistant Bacteria*Eadlapalli Siddhartha¹, Vemula Sarojamma², and Vadde Ramakrishna^{1*}*

¹Department of Biotechnology and Bioinformatics, Yogi Vemana University, Kadapa – 516003 (Andhra Pradesh) India, ²Department of Microbiology, Sri Venkateswara Medical College, Tirupathi – 517002 (Andhra Pradesh) India

Abstract:

Background: Multidrug Resistance (MDR) among bacteria become a global concern due to failure of antibiotics, is drawn attention for best antimicrobials from the spices which have been using ancient days in Indian culinary and traditional medicine. **Aim and Objectives:** The present study was undertaken to evaluate the bioactive compounds and their antibacterial activity in routinely used culinary Indian spices against β -lactamase produced MDR bacteria. **Material and Methods:** Ethanolic extracts prepared from twenty spices and were evaluated for total phenolics, flavonoids, alkaloids, terpenoids, antioxidant properties, and also assayed their antibacterial activities against β -lactamase producing MDR bacteria (*Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*). β -Lactamase and cell viability assays were performed in MDR bacteria. **Results:** Among twenty spices, cinnamon and clove exhibited highest levels of phenolics and terpenoids with elevated antioxidant potential and also showing greater reducing potential at lower concentrations of extract (2.3 and 4.06 μ g GAE/gm), respectively. Further, the spices extracts were assessed for antimicrobial activity against β -lactamase produced tested MDR bacteria and observed higher antimicrobial activity with cinnamon, garlic, tamarind and clove at lowest concentrations of MIC and MBC at 16 - 32 μ g GAE/ml, as compared to standard drug, amoxiclav (16/8 μ g/ml). Spices significantly inhibited the β -lactamase activity (80–94%) and also cell viability in tested MDR bacteria. **Conclusion:** Indian spices consist of rich

bioactive profile and antioxidant activity inhibited the bacterial growth effectively by suppressing β -lactamase production in MDR bacteria. Results indicating the spices as functional foods and could be used in prevention of antibiotic resistance.

Keywords: Spices, Bioactive Compounds, Antioxidants, Cell Viability, β -Lactamase Inhibition, Antibacterial Activity

Introduction:

Antibiotic resistance in bacteria becomes tough to treat and is causing a universal health emergency. It is estimated that 58,800 neonatal sepsis deaths occur in India due to drug resistant infections [1]. Nearly 25,000 and 23,000 public deaths are happening among Europe and United states, respectively every year due to the Multidrug Resistance (MDR) among bacteria [2]. According to the statistics, resistance among the common bacteria enhanced hugely during the period from 2008 to 2014 particularly in *E.coli* increased from 10 to 13%, in *K. pneumoniae* from 29 to 57%, *S. aureus* from 29 to 47% and *S. typhi* from 8 to 28% [1]. There are three major mechanisms involved in the antibiotic resistance among bacteria viz. minimizing entry of antibiotics through efflux pump or penetration, antibiotic inactivation by modification or by hydrolyzing it, and target site modification by mutation [2]. β -lactamases are the important enzymes which makes bacterial

resistance to β -lactam antibiotics and responsible for MDR. These are mostly seen in Enterobacteriaceae family, of which *E. coli* and *K. pneumoniae* are common species [3, 4]. The enzyme genes are co-located with other antimicrobial resistance determinants on plasmids, and rendering strains multidrug resistant [5]. The most common Extended Spectrum β -lactamases (ESBL) are Sulphydryl Variable (SHV), Temoneria (TEM), Cefotaxime (CTX), Oxacillin (OXA) classes of β -lactamases [4]. Outcome of the extensive use of antibiotics is the colonization of the compounds in livestock and environment, and this scenario plays a major role in the antibiotic resistance evolution due to the frequent exposure of different concentrations of drugs by the bacteria [6, 7]. Lack of awareness in public particularly overuse of antibiotics is responsible for increased occurrence of MDR in bacteria [1]. It is expected that by 2050, the drug-resistant infections will be the leading cause of deaths than the cancer [1]. To stop this situation there is an urgent need to identify potential antimicrobials with minimal side effects on the host cell. Increased usage of antibiotics is proportionately upsurges antibiotic resistance in bacteria, but there is no parallel increase in the discovery of new agents with considerable enhanced spectrum of antibiotic activity. No major class of new antibiotics were discovered since 1962, and even if is discovered they are modified versions of existing structures and still showing the side effects, short life expectancy and bacterial resistance [8, 9]. Hence, this alarms to discover the alternative, safe and potential antibiotics that may not give side effects, that should be cost effective and for which bacteria does not get resistant. In recent years, herbs and spices of traditional medicines gained special interest all around world

because of safe use, and good food perseverative characteristics since ancient days and also an important resource for discovery of new antibiotics [10, 11]. Now it is about 80% of the world population rely on botanical preparations as therapy to treat different infections without any side effects. Numerous studies were documented on the antimicrobial properties of medicinal plants [12- 15] but more vacuum noticed in literature on antibacterial activity of Indian spices even though they were used in routine culinary since ancient times as flavour and aroma enhancers, colourants, preservatives and traditional medicines [16]. India is the largest producer, consumer and exporter of spices around the world, and contributing 86% of global spice production followed by China (4%), Bangladesh (3%), Pakistan (2%), Turkey (2%) and Nepal (1%) [16]. Previously, some of the authors noticed on selected spices consisting of rich levels of phenolic compounds, and also noticed a linear relationship with antioxidant properties [17, 18]. Earlier studies reported antimicrobial nature of selected spices against normal bacteria [17-24]. However, to our knowledge, there are no studies available in literature about evaluation of bioactive components and their antibacterial activities in routinely used culinary Indian spices against ESBL produced MDR bacteria. Hence, the present study was aimed to evaluate bioactive compounds and antioxidant properties present in spices and also studied their antibacterial activities against ESBL producing MDR bacteria - *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*. We have noticed that higher levels of phenolics, and terpenoids in Indian spices exhibited elevated antimicrobial activities, which is parallel with standard antibiotic, axomiclov.

Material and Methods:

All routinely used chemicals and reagents were purchased from Merck, India, and Thermo fisher scientific company, India. Rutin, Trolox ((±)-6-hydroxy-2, 5, 7, 8-tetramethylchromane-2-carboxylic acid), linalool, ABTS (2, 2'azino bis (3-ethylbenzothiazoline 6-sulfonate sodium salt), gallic acid and INT (Iodonitrotetrazolium chloride) were purchased from the Sigma-Aldrich, USA. All antibiotics were purchased from the Himedia laboratories, India. Confirmed ESBL positive bacterial cultures were obtained from the tertiary care hospital, Anantapur, Andhra Pradesh, India, these are *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*.

Plant Material Collection:

Spices used in the study were bought from local market, identified and deposited in the herbarium, Dept. of Botany, Yogi Vemana University, Kadapa, India [17]. Selected spices were made to a fine powder and sieved using 0.2 mm sieve and stored until further use in room temperature in a sealed cover.

Extraction:

Extractions of spices were performed under ice cold conditions by taking 2 gm of spice powder dissolved in 20 ml of 80% ethanol and kept on a shaking incubator overnight at 10°C. The extract was centrifuged at 4000 rpm for 5 min, supernatant collected and pellet reused for extraction. The fresh solvent was added to the remained pellet and kept on shaking incubator for 3 h. It was repeated twice so that all the bioactives present in the powder will be diffused completely into the extraction solvent. Collected supernatants were pooled, concentrated in vacuum under reduced pressure by using Rota evaporator (Heidolph Rotary Evaporator, Germany). Concentrated

extracts were obtained, aliquoted and stored at -80°C until use [17, 25].

Total Phenolics Assay:

Total phenolics were estimated as described by Singleton *et al.* [26], with slight modification. To 140 µl of the extract or gallic acid standard, added 600 µl of the 0.2 M Folin-Ciocalteu reagent. After 5 min, 460 µl of 7.5% (w/v) sodium carbonate was added. The reaction mixture was incubated in dark at 45°C for 30 min, followed by one-hour incubation at room temperature. Absorbance was measured at 765nm against blank. Results were expressed as mg gallic acid equivalents per gram of spice (mg GAE/gm).

Alkaloids Assay:

Alkaloids levels determined by Novelli *et al.* [27], with slight modification. To 1 ml of extract, 5 ml of Bromo Cresol Green (BCG) was added in a separating funnel. Then 5 ml of 0.1 M phosphate buffer of pH 4.7 was added and shaken vigorously. BCG forms complex with alkaloids present in the extract. Later 5 ml of chloroform was added, this entire solution will turn to yellow. Coloured complex formed was separated carefully into separate test tubes and absorbance was read at 470 nm against a blank having chloroform and BCG without any extract. Atropine was taken as standard and the result was expressed as mg atropine equivalents per gram of spice (mg AE/gm).

Terpenoids Assay:

Terpenoids were estimated by using the protocol developed by Ghorai *et al.* [28]. The experiment was carried out in 2 ml microfuge tubes. To 200 µl extract, added 1.5 ml of chloroform, after 3 min 100 µl of conc. H₂SO₄ was added. Heat generated at this step was reduced by keeping the entire

setup on ice for less than 15 min, followed by incubation for 90 min to 120 min in the dark. Linalool was used as standard and it was not incubated for more than 5 min during the incubation period, the red precipitate was formed at the bottom of the tube. The supernatant was decanted carefully without disturbing the pellet. Pellet was dissolved by adding 1.5 ml of 80% methanol and absorbance was read at 538nm. Results were expressed as mg of Linalool equivalents per gram (mg LE/gm) of spice.

Flavonoids Assay:

The aluminum chloride colourimetric method [29] was used for flavonoid determination. To 25 µl of extract, 75 µl of 95% alcohol was added, followed by 5 µl of 10% aluminum chloride and 1M potassium acetate. Finally the volume was made up to 260 µl with distilled water. After 40 min incubation at room temperature, absorbance of the reaction mixture was measured at 415 nm. The calibration curve was prepared by taking rutin as standard and results expressed in mg of Rutin Equivalents per gram (mg RE/gm) of spice.

ABTS Antioxidant Assay:

ABTS assay was performed by following the protocol described by Re *et al.* [30]. Before 16 h of the experiment, mother solution of ABTS was prepared by mixing equal volumes of 8 mM ABTS and 3 mM potassium persulfate and allowed to react in dark condition for the generation of ABTS free radicals. Working solution of ABTS was prepared by adding 1 ml of the mother solution to the 29 ml of the 0.2 M phosphate buffer of pH 7.4. To 10 µl of the spice extract, 290 µl ABTS working solution was added and incubated at room temperature for 30 min. Then discoloration of ABTS solution was measured at 734 nm. Trolox was taken as the standard antioxidant and results expressed as mg of Trolox equivalents per gm (mg TE/gm) of the spice.

Reducing Power Assay:

Reducing power assay was performed by following method of Ferreira *et al.* [31]. Aliquots of 10, 20, 30 and 40 µl of the extract were taken in 5 ml test tubes and volume was made up to 400 µl with distilled water, To this 500 µl of 0.2 M phosphate buffer of pH 6.6 and 500 µl of 1% potassium ferricyanide was added, and kept in the water bath at 50°C for 20 min. Later, tubes were removed and added 10% trichloroacetic acid to stop the reaction. Tubes were centrifuged at 3500 rpm for 10 min and collected 0.5 ml of upper layer and mixed 0.5 ml of distilled water and 100 µl of 0.1% ferric chloride. Absorbance was read at 700 nm against blank. Ascorbic acid was taken as standard and results expressed as EC50, where EC50 stands for effective concentration at which absorbance was 0.5.

Determination of Antimicrobial Activities of Spices:

Antibacterial activity was performed by disc diffusion method developed by Bauer *et al.* [32]. Ethanolic extracts of the spices were evaluated for antibacterial activity against three Gram-negative ESBL produced bacteria *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and one Gram-positive bacteria *Staphylococcus aureus*, which were obtained from tertiary care hospital. These bacterial samples are ESBL confirmed clinical isolates.

Inoculum preparation:

Loop full of the 24 h culture of respective bacteria was taken and dropped in 5 ml of nutrient broth. Tube was incubated in a shaking incubator at optimum temperature until culture reaches the 0.5 McFarland units which have bacterial concentration of 1.5×10^8 CFU.

Disc diffusion method:

Bacterial culture was spread over the Muller-Hinton agar plate with a sterile cotton swab. At four corner of the seeded petri plate, Whatmann No.1 filter paper discs of size (6 mm) were placed. Thirty μl ethanol extract(s) of Indian spices were dropped over discs. The entire set up was incubated at 37°C for 24 h, the Zone Of Inhibition (ZOI) was measured after incubation and expressed results in mm of ZOI. Amoxiclav (16 μg Amoxicillin and 8 μg of Clavulanic acid) was used as the standard antibiotics.

Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC):

MIC was performed as per the American Society of Microbiology (ASM) manual according to CLSI guidelines and by Eloff 1998 [33]. MIC and MBC of spice extracts were determined as follows. To 1 ml of the nutrient broth, added different concentrations of spice extract(s). Log phase cultures of bacteria were taken and checked the absorbance of the culture in the spectrophotometer (0.6 to 0.8 OD values 1.5×10^8 CFU/mL). Culture was diluted 20 times and taken 100 μl of the diluted culture, added directly to the tubes and incubated for 16 h. Then 40 μl of 0.2 mg/ml INT was added to the tubes and incubated at 37°C for 30 minutes. After incubation, colour change (colourless or yellow to pink) was noticed in tubes indicating bacterial growth and no colour change in the tubes indicating absence of bacterial growth, the concentration where no colour change is considered as MIC concentration.

For the determination of the MBC, a portion (10 μl) of the above MIC experimental solution from the tube at which no colour change noticed was taken and spread over the sterile Muller Hinton agar plate, incubated for 24 h for cell

growth. The plate in which no bacterial colonies observed was considered as MBC concentration. MIC and MBC were expressed as μg GAE of extract/ml.

Cell viability assay:

To determine the time at which concentration of extract inhibits bacterial cell growth; cell viability assay was performed by following the method of Arora and Kaur [19]. To 1 ml of the fresh broth, 100 μl of 10^6 CFU bacterial cultures was added. Subsequently, spice extract at MBC concentration was added to the suspension and incubated at 37°C . At different time intervals (0 h, 1 h, 2 h, 3 h, 4 h, and 8 h) 20 μl of the suspension was taken out and spread over nutrient agar plates and incubated at 37°C for 16 h and observed for the growth of bacterial colonies. The mean number of colonies counted and compared with that of the control in which extract was replaced with sterile distilled water. The results were expressed in viable cells as a percentage of inhibition of growth in respect to control.

$$\% \text{ of inhibition of growth} = \left(1 - \frac{\text{Test}}{\text{Control}} \right) \times 100$$

-lactamase assay:

-lactamase inhibitory activity of spice extract was performed by Livermore and Brown [34]. To bacterial suspension of 10^6 CFU, penicillin at 6mg/ml in phosphate buffer of pH 6.0 or spice extract at MBC concentration was added and incubated for 16 h at 37°C . Later to this suspension, added 20 μl of 1% (w/v) soluble starch, followed by 20 μl of 2% (w/v) iodine in 53% (w/v) aqueous potassium iodide. After 5 min, decolourization of the suspension indicates the negative -lactamase activity of spices (i.e. spices did not inhibited the beta-lactamase enzyme) and

formation of the blue colour indicates positive - lactamase activity of spices (i.e. spices inhibited the beta-lactamase enzyme). Results were expressed as the percent of the beta-lactamase inhibition by using the formula.

$$\% \text{ of relative enzyme activity (REA)} = \left(\frac{\text{Test}}{\text{Control}} \right) 100$$

$$\% \text{ of enzyme inhibition} = (100 - \text{REA})$$

Statistical analysis:

Data were represented as Mean \pm Standard Error (SE) of each value of five independent determinations in the tables and figures, unless otherwise stated. Statistical significance was determined by one-way ANOVA.

Results:

Indian spice(s) were extracted with 80% ethanol and used for analysis of bioactive compounds (total phenolics, terpenoids, alkaloids, and flavonoids), antioxidant activities, and their antibacterial activity against ESBL produced MDR bacteria to identify an effective spice involved in antibacterial activity. The extract yield was variable from one spice to other (tamarind – 13% to onion – 5%).

Enriched bioactive compounds of Indian spices exhibited high antioxidant activities with elevated reduction potential

Ethanol extracts of Indian spices were evaluated for total phenolics, flavonoids, alkaloids, and terpenoids and observed substantial levels of these bioactives in all spices used in the study. The results were depicted in Table 1. Much variation was noticed among spices. Higher amount of the phenolics noticed in bay leaf (195 mg GAE/gm), followed by clove (188 mg GAE/gm), cinnamon (168 mg GAE/gm) and least phenolics were seen in onion (0.13 mg GAE/gm). Terpenoids were also varied greatly among tested spices more terpenoids were seen in clove (319.2 mg LE/gm)

followed by cinnamon (255.7 mg LE/gm) and less terpenoids were noticed in onion (21.2 mg LE/gm). The levels of flavonoids observed in a range from 31.9 mg RE/gm in turmeric to 0.15mg RE/gm in garlic with moderate levels in cinnamon, clove, and ajowan. However, the levels of alkaloids found in spices are very low, ranged from 2.26 mg AE/gm in mustard to 0.004 mg AE/gm in onion. Due to the presence of the high levels of total phenolics and terpenoids in experimental spices, they exhibited the profound antioxidant activity (Table 1). With higher levels of total phenolics, and terpenoids in clove, and cinnamon showed the highest ABTS free radical scavenging activity (572 and 319 mg TE/gm) with efficient reducing potential at very low concentration of extract - 2.3 and 4.06 μ g GAE/gm extract, respectively. Similarly, bay leaf showed higher levels of total phenolics (195 μ g GAE/gm) as well as terpenoids (128 mg LE/gm) with elevated antioxidant activity (249 mg TE/gm), but reducing potential of extract was high (45 μ g GAE/gm) as compared to low level in clove and cinnamon. Remaining spices like garlic, star anise, and ajowan exhibited the moderate levels of total phenolics, and terpenoids with good antioxidant potential. However, in tamarind fruit pulp extract, we noticed the lower levels of total phenolics (12 mg GAE/gm) and higher levels of terpenoids (160 mg LE/gm) with good ABTS free radical scavenging activity (79 mg TE/gm) and exhibited efficient reduction potential at the concentration 10 μ g/gm. Similar results noticed in staranise, however, reducing potential of the extract was 3.38 μ g GAE/gm. The garlic showed higher levels of terpenoids (144 mg TE/gm) with good ABTS free radical scavenging activity (91.2 mg TE/gm) and exhibited efficient reducing potential at lower concentration 8.22 μ g GAE/gm. However, the extracts of onion, curry leaf, and ginger were showed least levels total phenolics as well as

Table 1: Bioactive Compounds and Their Antioxidant Status in Ethanolic Extracts of Routinely Used Indian Spices

Plant Name	Biological Source	Part used	Phenolics mg GAE/gm (n=5)	Flavonoids mg RE/gm (n=5)	Terpenoids mg LE/gm (n=5)	Alkaloids mg AE/gm (n=5)	ABTS mg TE/gm (n=5)	Reducing Potential (EC ₅₀ , µg GAE/gm) (n=5)
Ajowan	<i>Trachyspermum ammi</i> S.	Seed	26.4 ± 0.37	5.3 ± 0.17	84.2 ± 11	0.84 ± 0.06	127.3 ± 0.91	12.78 ± 1.2
Bay leaf	<i>Laurus nobilis</i> L.	Leaf	195.4 ± 4.1	6.2 ± 0.14	128 ± 13.7	0.08 ± 0.004	249 ± 1.2	45.38 ± 2.6
Black Pepper	<i>Piper nigrum</i> L.	Fruit	5.86 ± 0.03	15 ± 0.69	65 ± 6.5	0.9 ± 0.06	49.2 ± 0.6	11.60 ± 0.8
Caper	<i>Capparis spinose</i> L.	Bud	59 ± 0.9	3 ± 0.08	254.6 ± 20.1	0.14 ± 0.03	273 ± 5.7	22.19 ± 1.3
Cardamom	<i>Elettaria cardamom</i> L.	Seed	0.96 ± 0.1	1.45 ± 0.12	71.16 ± 3.5	0.89 ± 0.06	38.2 ± 0.3	15.16 ± 0.21
Chilli Pepper	<i>Capsicum frutescens</i> L.	Fruit	6.8 ± 0.05	9 ± 0.09	71.7 ± 6.7	0.19 ± 0.06	83.9 ± 0.2	21.5 ± 0.9
Cinnamon	<i>Cinnamomum zeylancium</i> B.	Bark	168.2 ± 1.4	4.14 ± 0.39	255.7 ± 16.6	0.99 ± 0.05	319.8 ± 7.5	4.06 ± 0.3
Clove	<i>Syzygium aromaticum</i> L.	Bud	188.4 ± 2.3	12.8 ± 0.38	319.2 ± 6.6	0.34 ± 0.02	572.9 ± 7.5	2.3 ± 0.09
Coriandrum	<i>Coriandrum sativum</i> L.	Seed	2.4 ± 0.08	1.91 ± 0.63	37.1 ± 3.9	0.47 ± 0.03	18.9 ± 0.7	13 ± 0.7
Cumin Seeds	<i>Cuminum cyminum</i> L.	Seed	9.6 ± 0.07	12.6 ± 0.1	73 ± 5.6	1.5 ± 0.06	76.9 ± 3.4	14.69 ± 1.4
Curry leaf	<i>Murraya koenigii</i> L.	Leaf	5.5 ± 0.23	3.2 ± 0.04	31.5 ± 2.1	0.33 ± 0.02	25.9 ± 0.15	21.29 ± 1.7
Garlic	<i>Allium sativum</i> L.	Bulb	0.16 ± 0.02	0.15 ± 0.01	144.6 ± 1	0.012 ± 0.001	91.1 ± 0.1	8.22 ± 0.6
Ginger	<i>Zingiber officinale</i> R.	Rhizome	1.06 ± 0.01	0.88 ± 0.03	34.9 ± 1.8	0.15 ± 0.05	15.1 ± 0.2	14.22 ± 0.9
Mustard	<i>Brassica juncea</i> L.	Seed	3.69 ± 0.05	1.86 ± 0.1	43.7 ± 3.2	2.26 ± 0.1	34.2 ± 0.57	8.52 ± 1.3
Onion	<i>Allium cepa</i> L.	Bulb	0.13 ± 0.003	0.23 ± 0.04	21.2 ± 0.87	0.004 ± 0.001	9.7 ± 0.03	22.12 ± 2.9
Star anise	<i>Illicium verum</i> H.	Fruit	157 ± 1.1	2.3 ± 0.1	195.5 ± 1.87	0.49 ± 0.03	82 ± 0.28	3.38 ± 0.5
Tail Pepper	<i>Piper cubeba</i> L.	Fruit	7.88 ± 0.19	9.4 ± 0.1	80.5 ± 4.96	1 ± 0.006	87.3 ± 0.38	11.94 ± 0.1
Tamarind	<i>Tamarindus indica</i> L.	Fruit pulp	12 ± 0.01	2 ± 0.02	160 ± 3.49	0.17 ± 0.01	79.6 ± 0.56	10.4 ± 0.57
Trigonella	<i>Trigonella foenum gracum</i> L.	Seed	4.3 ± 0.16	7.6 ± 0.33	85.69 ± 11.42	0.85 ± 0.04	72.9 ± 2.1	20.46 ± 1.5
Turmeric	<i>Curcuma longa</i> L.	Rhizome	17.2 ± 0.07	31.9 ± 1	81.24 ± 6.5	0.72 ± 0.009	121.8 ± 0.9	25.55 ± 0.8

Note: GAE - Gallic acid equivalents, RE - Rutin equivalents, LE - Linalool equivalents, TE - Trolox equivalents, AE - Atropine equivalents, EC₅₀ - Half maximal Effective concentration, ND - Not detected. Each value presented in table represents the Mean ± SE of five independent determinations.

terpenoids with lower capacity of antioxidant potential.

Indian spices suppresses the growth of ESBL produced multidrug-resistant bacteria

Ethanollic extracts of Indian spices were evaluated for antibacterial activity against four clinical isolates of MDR bacteria, of which three were Gram negative and ESBL positive bacteria (*Escherichia coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*) and one of Gram positive bacteria Methicillin Resistant *Staphylococcus aureus* (MRSA) confirmed as per CLSI guidelines. Great variation was seen in antibacterial activities of all tested spices against above bacteria as compared to standard control drug (Axomiclov). High antibacterial activity (15-20 mm) was noticed with the extracts of garlic, cinnamon, tamarind, clove and ajowan against tested bacteria in terms of ZOI as compared to drug control (21-23 mm). However, this growth inhibition was observed at very low concentrations of ethanolic extract (range 16-128 µg GAE/ml) as compared to drug control values (16/8µg/ml), which were determined through MIC and MBC for tested spices. Ajowan and star anise extracts showed moderate inhibitory activity against all tested bacteria at 128 µg GAE/ml of MIC and MBC. Among tested spice extracts, the cinnamon extract exhibited highest antibacterial activity against all tested multidrug resistant bacteria at lowest concentration of MIC and MBC extract at 16 µg GAE/ml, followed by garlic at 16 and 32 µg GAE/ml. However, the pulp of tamarind extract exhibited higher antimicrobial activity at 32 and 64 µg GAE/ml of MIC and MBC only against *E. coli* and *S. aureus* as compared to other bacteria. These results depicted in Table 2.

Onion, mustard and trigonella extracts did not shown growth inhibition against all tested bacteria even at higher concentration up to 1.0 mg GAE/ml. However, coriander, curry leaf, bay leaf, caper and ginger extracts were shown less growth inhibition activity at higher values of MIC and MBC. Results achieved in the present study shown that studied spices hold impending antibacterial activity against entire tested organisms.

Indian spices inhibited the cell viability of multidrug-resistant bacteria

Cell viability assay was performed to know the effect of extract on the viability of tested bacterial cells and also the time at which the spice extract inhibits growth completely. It is observed that the cinnamon at its MBC (32 µg GAE) inhibited all the tested MDR bacterial cell viability exorbitantly (100%) after 3h of incubation. MBC of garlic extract (32 µg GAE) inhibited viability completely in *E. coli* and *K. pneumoniae* after 3h of incubation, but *P. aeruginosa*, and *S. aureus* bacteria cells were still viable (15 – 20%) at this time. Similar results were noticed on treatment with tamarind extract at its MBC (62 µg GAE). The cell viability results were depicted in Fig. 1. Treatment of ajowan extract (MBC, 256 µg GAE) exhibited 100 % loss of cell viability against *E. coli* and *K. pneumoniae* in 3h of incubation and took 6 h for *P. aeruginosa* and *S. aureus*. However, the clove extract (MBC, 256 µg GAE) inhibited cell viability of all tested organisms only after six hours of incubation. Onion, trigonella, peppers, and mustard did not shown cell viability inhibition; however, we noticed an increased number of colonies in these extracts treated plates.

Table 2: Antibacterial Activity, Minimum Inhibitory Concentration and Minimum Bactericidal Concentration of Ethanolic Extracts of Indian Spice(s) against ESBL Producing Multidrug Resistant Bacteria

Plant Name	ESBL Positive Bacteria						MRSA Bacteria					
	<i>E. coli</i>			<i>K. pneumoniae</i>			<i>P. aeruginosa</i>			<i>S. aureus</i>		
	ZOI (n=5)	MIC (n=5)	MBC (n=5)	ZOI (n=5)	MIC (n=5)	MBC (n=5)	ZOI (n=5)	MIC (n=5)	MBC (n=5)	ZOI (n=5)	MIC (n=5)	MBC (n=5)
Ajowan	12 ±0.2	128	256	15 ±0.7	128	128	20 ±1.4	128	128	13 ±1	128	256
Bay leaf	9 ±0.3	256	512	10 ±0.6	256	512	9 ±0.3	512	512	10 ±0.3	256	512
Black Pepper	12 ±0.6	256	512	ND	ND	ND	10 ±0.4	256	512	9 ±0.8	512	512
Caper	13 ±0.3	256	512	12 ±0.3	512	512	10 ±0.2	512	ND	9 ±0.4	512	512
Cardamom	8 ±0.1	512	512	8 ±0.2	256	512	12 ±0.8	256	512	12 ±0.6	256	512
Chilli Pepper	ND	ND	ND	10 ±0.5	256	512	10 ±0.7	256	512	9 ±0.5	ND	ND
Cinnamon	15 ±0.8	16	32	15 ±0.7	16	16	18 ±1.5	16	16	15 ±1.2	16	16
Clove	13 ±0.4	128	256	15 ±0.5	256	512	15 ±0.6	256	512	15 ±0.8	128	256
Coriandrum	ND	ND	ND	11 ±0.3	512	512	9 ±0.3	512	512	11 ±0.7	512	512
Cumin Seeds	10 ±0.6	512	512	10 ±0.2	512	ND	9 ±0.3	512	512	13 ±1.3	128	256
Curry leaf	9 ±0.5	512	512	10 ±0.5	512	512	11 ±0.4	512	ND	10 ±0.6	512	512
Garlic	20 ±1.2	16	32	15 ±0.8	16	32	15 ±0.8	16	32	14 ±1	16	32
Ginger	ND	ND	ND	10 ±0.4	512	512	ND	ND	ND	ND	ND	ND
Mustard	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Onion	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Star anise	12 ±0.8	256	512	14 ±0.7	128	256	15 ±1.1	128	256	17 ±1.4	128	256
Tail Pepper	ND	ND	ND	ND	ND	ND	ND	ND	ND	12 ±1.1	256	512
Tamarind	18 ±0.9	32	64	16 ±0.3	64	128	17 ±0.9	64	128	17 ±0.8	32	64
Trigonella	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Turneric	11 ±0.3	256	512	12 ±0.4	256	512	10 ±0.9	256	512	9 ±0.7	512	512
Amoxiclav	21 ±0.4	16/8	32/16	23 ±1.3	16/8	32/16	22 ±1.5	16/8	32/16	22 ±1.5	16/8	32/16

Note: ZOI – Zone of inhibition in mm, MBC – minimum bactericidal concentration in µg GAE of extract, MIC - minimum inhibitory concentration in µg GAE/ml of extract, ND – Not detected even at 1mg GAE/ml of extract. Each value presented in table represents the Mean ± SE value of five independent determinations.

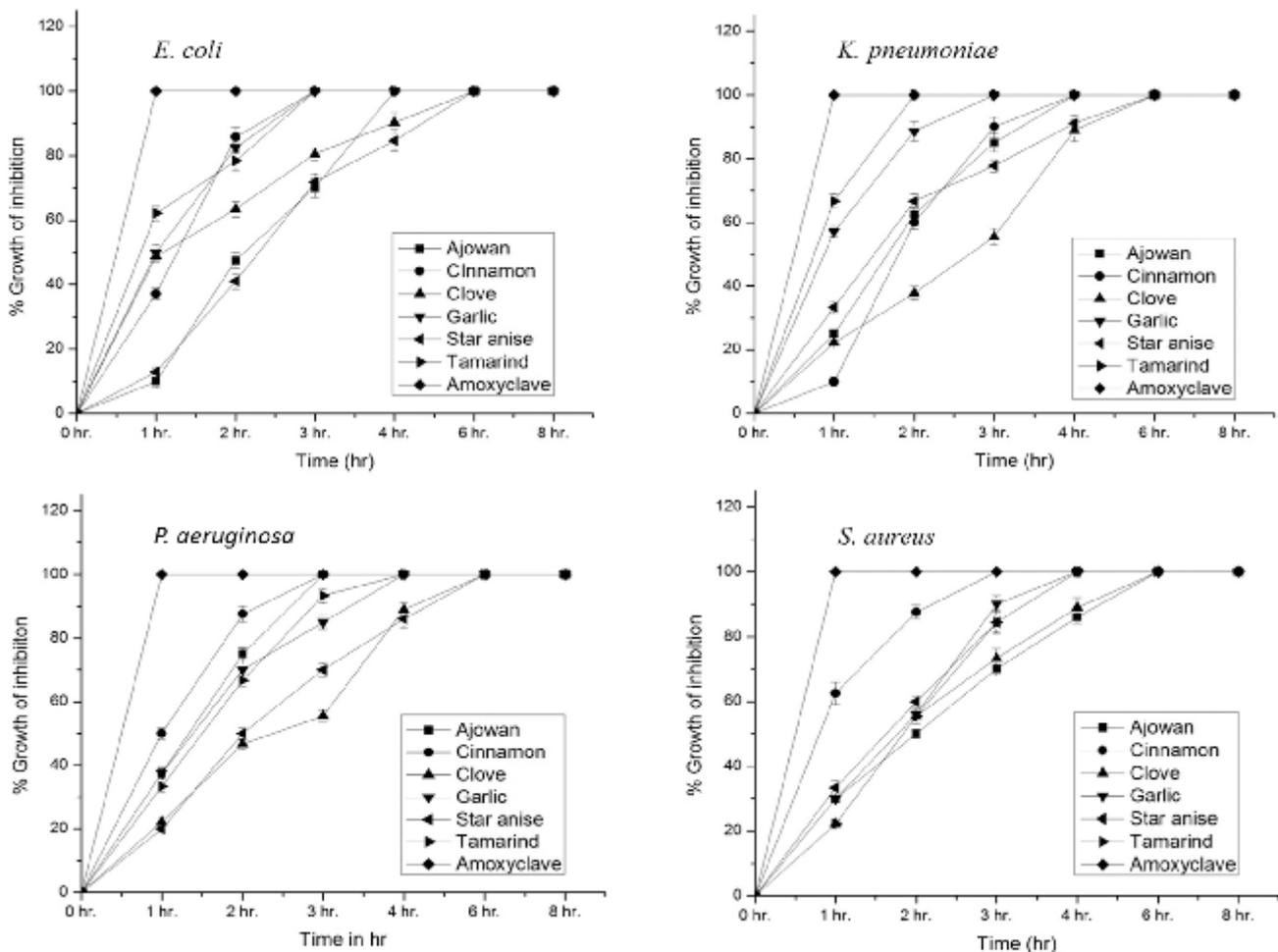


Fig. 1: Changes of Cell Viability of *E. coli*, *K. pneumoniae*, *P. aeruginosa*, and *S. aureus* Bacterial Cells on Exposure of Indian Spice Extracts at Their Respective MBCs During 8 h Incubation

Indian spices inhibited the β -lactamase activity of multidrug-resistant bacteria

As we noticed a remarkable antibiotic activity in many of the spices used in study was prompted us to know whether these spices effectively inhibiting β -lactamase in all tested bacteria because these enzymes are primarily responsible for antibiotic resistance. All MDR bacteria were treated with spice extract(s) at their respective MBCs and performed iodometric assay. The results revealed that cinnamon treatment inhibited

88-90% of β -lactamase activity as compared to control (activity without treatment) in all tested bacteria and this inhibition was almost similar to the standard drug, amoxyclave. Similarly, β -lactamase inhibitions noticed in bacteria on treatments of garlic was 75-83%, tamarind 66-77%, ajowan 65-75%, clove 59-70% and for star anise 55-73% as compared to control drug. Curry leaf, onion, mustard, and ginger showed very less inhibitory activity (<10%) against all the tested bacteria (data not shown). The results were

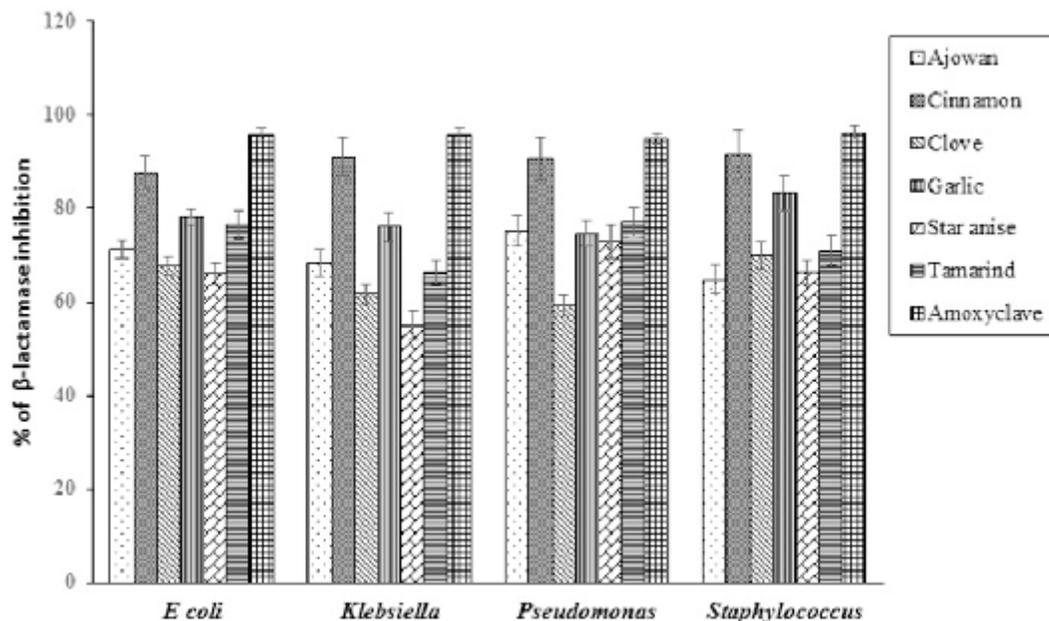


Fig. 2: Changes in the Levels of β -lactamase Activities on Exposure of Spice(s) Extracts against *E. coli*, *K. pneumoniae*, *P. aeruginosa*, and *S. aureus* Bacterial Cells.

represented in fig. 2. Overall, similar trend of β -lactamase activities noticed in *E. coli*, *P. aeruginosa*, and *S. aureus* bacteria; however, in *K. pneumoniae* the inhibition was lesser as compared to other bacteria.

Based on the results obtained from all above experiments of bioactive compounds, antioxidant potential and antibacterial activities, it was noticed that cinnamon, garlic, tamarind, ajowan and clove spices exhibited potential antimicrobial activity against tested ESBL produced multidrug resistant bacteria with their enriched phenolics and terpenoids and their antioxidant potential.

Discussion:

Due to the continuous failure of the drugs against antibiotic resistance among bacteria, there is an urgent need to overcome the problem. Hence, researchers are turning towards the plant products

in search of the novel compounds for the treatment of the drug resistant bacteria. Use of plant material in the form of food with known antimicrobial properties can be of great importance in this respect. Nevertheless, it is becoming strong that there is a huge band of the plant's metabolites that has not yet been described. Within these unknown and unidentified compounds there will be key defense components in both regulatory and unique antibiotic activities [35]. In the present study the spices displayed enriched bioactive compounds namely total phenolics, and terpenoids with enhanced antioxidant activities (Table 1). Our results were in line with the previous reports of chilli pepper [36], clove and black pepper [18], cinnamon, cumin and curry powder [37], tamarind [38], star anise and cinnamon [39], bay leaf [40], and garlic [17].

These enriched bioactive compounds of spice extracts are probably responsible for antibiotic activity. The outcome of antibacterial activities through disc diffusion, MIC and MBC results is presented in table 2 that spices had effective and reliable inhibitory effect against the tested ESBL produced multidrug resistance bacteria. Relatively a lot of hydroxyl groups are present in the phenolic compounds in herbal extract which forms the hydrogen bonds with the electro-negative atoms in proteins and nucleic acids and interrupt the function [41]. Terpenoids interact with the membranes and increase the membrane fluidity and permeability which leads to the uncontrolled efflux of ions and metabolites from the cell and finally undergo necrotic or apoptotic cell death [41]. From the literature we came to know that there were no reports on the total terpenoids, alkaloids and flavonoids of the spices, and it is the first study reporting these compounds quantitatively. Cinnamon, garlic, tamarind, clove and ajowan showed high antibacterial activity against all tested bacteria as compared to drug control, axomiclov. Previous reports suggested that the major compounds present in cinnamon and garlic are cinnamaldehyde and allicin which acts on the bacterial membrane and initiates the cellular leakage leading to the cellular death [42]. Beta-lactamase activity, predominant enzyme involved bacterial drug resistance, in all tested bacteria were profoundly inhibited by cinnamon, garlic, tamarind, ajowan and clove very effectively. It is pertinent to note that bioactive compounds rich spices inhibited the activity of beta lactamase. Inhibition of growth of these bacteria through terpenoids damages membrane and phenolics, inhibiting enzyme activity and

finally make susceptible for bacterial cell death. The results of the current study are revealed that nine spices (cinnamon, clove, tamarind, ajowan, garlic, star anise, bay leaf, cardamom and turmeric) out of twenty tested showed significant antibacterial properties against all tested bacteria. Previously similar studies were reported on the antimicrobial activity of the medicinal plants (*Tectona grandis*, *Euphorbia hirta*, *Terminalia arjuna*, *Alstonia macrophylla*, *Claoxylon indicum*, *Pedilanthusti thymaloides*, *Citrus grandis*, *Citrus hystrix*, *Citrus reticulate* etc.) and few selected spices (garlic, ginger, onion, coriandrum, black pepper, green chillies, and cardamom) against normal bacteria [12-15, 43]. Arora and Kaur [12] reported that ajowan, and cardamom exhibited antimicrobial activity against *E.coli*, but cinnamon and clove didn't show activity against *E. coli* whereas, in the present study, cinnamon and clove revealed potential activity. A study conducted by Rahman *et al.* [43] on *E. coli* isolates reported that no spices showed the activity alone, however, noticed very little activity with synergistically. The present study is revealed the high antibacterial activity noticed with the extracts of garlic, cinnamon, tamarind, clove and ajowan as compared to drug control. The cell viability assay conducted by Arora and Kaur [19] revealed that garlic and clove effectively inhibited the cell viability after addition of extract on the tested bacteria. These results are in line with present study that the cinnamon inhibited all the tested MDR bacterial cell viability immensely (100%) within 3 h of incubation. Garlic extract inhibited viability completely in *E. coli* and *K. pneumoniae*, but *P. aeruginosa*, and *S. aureus* bacteria cells were still viable (15-20%). The -lactamase

inhibition activity obtained by Shaikh *et al.* [44] were different from the results of present study, in their study ginger showed activity with 25% enzyme inhibition which was maximum among all the tested spices. However, the results of antibacterial activity performed through disc diffusion method against *Staphylococcus aureus* were similar with the present study. Similar studies conducted by Akkiraju *et al.* [45], garlic inhibited the β -lactamase completely and ginger didn't inhibit the β -lactamase. These results are in line with the present study.

Conclusion:

Routinely used Indian culinary spices displayed good antimicrobial activity against β -lactamase produced MDR bacteria with their enriched phenolics, terpenoids and their antioxidant capabilities. The present research affords support to the research community in finding the appropriate treatments for drug resistance bacteria. Nevertheless, much research needs to be happening to authorize the mechanism of action of spice extracts and the compounds present in them.

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**Author for Correspondence: Dr. V. Ramakrishna, Department of Biotechnology & Bioinformatics,
Yogi Vemana University, Kadapa – 516003. Andhra Pradesh, India.
Email: vrkrishna70@gmail.com, vrkbtbi@yogivemanauniversity.ac.in Cell: 9866016961*