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PLANTS DERIVED EFFLUX PUMP INHIBITORS: AN APPROACH AGAINST MULTIDRUG-RESISTANT GRAM-NEGATIVE BACTERIA *KLEBSIELLA PNEUMONIAE*

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ABSTRACT

The AcrAB/TolC efflux pump-mediated drug resistance in gram-negative bacteria might be controlled by efflux pump inhibitors (EPIs). This study was planned to discover a compound from plants that can be used as an EPI. Forty isolates of *K. pneumoniae* and 33 different plants were collected. The methanolic extracts of parts of plants were prepared. Chloramphenicol, tetracycline, and Ciprofloxacin were used for the sensitivity assay. Plant extracts were tested for EPI activity using Berberine and Ethidium bromide assays. Only 2/40 isolates of *K. pneumoniae* were found to contain MDR-containing AcrAB-TolC efflux pumps. In 8/33, EPI activity was observed. *T. chebula* extract had maximum EPI activity and inhibited K. pneumoniae growth in vitro. Compounds were extracted and characterized from *T. chebula*. The compounds were tested against *K. pneumoniae* in combination with tetracycline, ciprofloxacin, and chloramphenicol. Ethyl gallate (C9H10O5) isolated from *T. chebula* has EPI activity against MDR *K. pneumoniae*. Compounds like ethyl gallate inhibit the efflux pump in bacteria and are highly effective in killing bacteria even at very low concentrations since they inhibit the efflux pump. Ethyl gallate is a novel Efflux pump inhibitor that when co-administered with antibiotic compositions helps to overcome drug resistance.

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Introduction

Antimicrobial resistance among microorganisms is an alarming and increasing problem throughout the world [1-3]. The widespread use of antibiotics led to the development of multi-drug resistant (MDR) organisms, nullifying the action of drugs formerly considered highly active. Infections involving MDR bacteria are a significant concern for most hospitals and healthcare facilities since they contribute not only to an increase in morbidity and mortality compared to the underlying diseases alone but also have an impact on the length of stay and associated healthcare costs. There are various drugs such as aminoglycosides, cephalosporin, and tetracycline for the treatment, but due to increased resistance against them, there is a need to develop either new drugs or restore the sensitivity of the currently available cornerstone drugs. MDR among bacteria may develop due to either of these two mechanisms: first, these bacteria may accumulate multiple genes, each coding for resistance to a single drug, within a single cell. This accumulation occurs on-resistance (R) plasmids, typically, and secondly, multi-drug resistance may also occur by the increased expression of genes coding for multi-drug efflux pumps, extruding a wide range of drugs [4]. These efflux pumps efflux out the drugs to decrease their concentration inside the cytoplasm [5].

The majority of gram-negative Bacterial efflux pump families, such as *K. pneumonia*, reduce antibiotic intracellular concentrations, resulting in intrinsic or acquired resistance to a variety of antibiotic classes [6-10]. Drug resistance to chloramphenicol, fluoroquinolones, tetracyclines, glycylcyclines, and florfenicol is caused by efflux pumps belonging to the MFS (Cml and Flo) and RND families (AcrAB-TolC, Acr AB, Mex XY, OprM, MexABOpM, MexCD-OprJ, Oqxb). Efflux pump-mediated drug resistance is an increasing problem, and to overcome this problem, the development and discovery of efflux pump inhibitors is an urgent necessity.

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EPIs are the substances that offer the most promising approach to blocking efflux pumps. They are the molecules that interfere with the process of removing toxic substances and antibiotics from the bacterial cell. Efflux pump inhibitors act as adjuvants to potentiate the activities of conventional medicines by inhibiting them either competitively or non-competitively [11, 12]. Several compounds have been discovered which are used as Efflux Pump Inhibitors (EPIs), as adjuvants, or in combination with antibiotics [10, 13-15]. During the trials of EPIs, they were found to be highly toxic and less effective. It is also essential to take into account both the pharmacokinetics and pharmacodynamics when choosing the efflux inhibitors to be adapted with those combined antibiotics [16]. Also, more techniques were required to design and quantify the EPIs and measure their kinetic parameters in the efflux pump components. These parameters were necessary for choosing between the general EPI that can stop the action of one transporter that expels various antibiotics in one species of bacteria or a specific EPI that stops the pumping of one antibiotic family in many bacteria [17, 18].

Plant-derived EPIs are synergistic enhancers of drugs. Though they may not have any antimicrobial properties alone, when they are taken concurrently with standard drugs, they enhance the effect of that drug [19]. Therefore, efflux pumps are viable antibacterial targets, and the development of potent efflux pump inhibitors is a promising and valid strategy for restoring the susceptibility of resistant strains of bacteria to antibiotics that are substrates of efflux pumps. Therefore, the present study was planned to discover the bioactive molecules that have efflux pump inhibitory activity for MDR strains of gram-negative bacteria from natural resources by screening medicinal plants.

Materials and Methods

Ethical Approval

Research work was approved by the Institute Ethical Committee vide letter number SUIEC/13/27 dated April 10, 2013.

Collection of Plant Material

Plants were collected from the University of Horticulture and Forestry Nauni, Solan (H.P.), Arya Vastu Bhandar Dehradun (Uttrakhand), and Shoolini University, Solan. Plants collected from the Arya Vastu Bhandar and Shoolini University were authenticated by Y. S. Parmar University of Horticulture and Forestry, Nauni, Solan (H.P.), India with farm vide receipt no. 047 and book no. 2915. A total of 33 medicinal plants *Terminalia chebula, Syzygium aromaticum, Syzygium joabolanum, Thymus vulgaris, Punica granatum, Tectona grandis, Hemidesmus indicus,Ficus bengalensis, Pterocarpus marsupium* Roxb, *Melissa officinalis, Thespesia populnea, Pelargonium graveolens, Centella asiatica,Citrus aurantium,Glycyrrhiza glabra, Rosemarinus officinalis, Myristica fragrance, Brassica nigra, Trigonella foenumgraceum, Sesamum indicum, Cuminum cyminum, Rhytididelphus squarrosus, Murraya koenigii, Trachyspermum ammi, Foeniculum vulgare, Nigella sativa, Curcuma longa, Zingiber officonale, Mentha piperita,Punica granatum, Alium sativum, Alium cepa, Pelargonium, Graveolens* were collected, which belong to different families and have potent medicinal properties. They are also known for their synergistic activity with varying antibiotics against various bacteria.

Drug-Resistant Strains

Forty isolates of *K. pneumoniae* were collected from Gian Sagar Medical College and Hospital, Rajpura, Distt. Patiala, Punjab (India). The isolates were inoculated in glycerol stocks and transported to the laboratory. All isolates were characterized in the immuno-parasitology lab at Shoolini University, Solan to identify multidrug-resistant strains. Informed consent was obtained from the individuals before taking the samples.

Control (Drug-Resistant and Sensitive) Klebsiella Pneumoniae Strains

The drug-resistant strain of *K. pneumoniae* AcrAB efflux pump repressor gene knockout strain (1740), (resistant strain), AcrAB efflux pump regulator gene knockout strain (1739), (sensitive strain) and AcrAB wild type strain, (52145; resistant strain) was obtained from Dr Erique Loblet, CIBERES, Bunyola, Spain and sensitive control strains and *K. pneumoniae* AcrAB efflux pump overexpressing strain, (KLPN86 and 105; resistant strain), was obtained from Dr. Mazzariol of Verona University, Italy, and one susceptible strain of *K. pneumoniae* (MTCC 109) was procured from the Institute of Microbial Technology, Chandigarh, India.

Cultivation of K. Pneumoniae

Cultures of 40 clinical isolates and controls were grown in nutrient broth and then stored in 15 % and 40% glycerol stocks until used. Furthermore, cultured isolates were kept alive by subculturing them in nutrient agar slants every 2 or 3 weeks.

Antibiotic Sensitivity Assay

All 40 clinical isolates were tested for antibiotic sensitivity. The antibiotic discs were of different concentrations, *i.e.*, 30 µg of tetracycline, 5 µg of ciprofloxacin, and 30 µg of chloramphenicol were used. Results were interpreted as resistant, sensitive, or intermediate as per the guidelines of CLSI (2006) [20]. Further, the screening of the MDR phenotype was performed by disc diffusion assay [21] using two groups of antibiotics, group A (Amikasin, Genetmycine, Ciprofloxacine, Chlroamphenicol, Tetracycline, Cefotaxim) and group B antibiotics (Cefdinar, Cefemine, Ertapenem, Imipenem, Meropenem, Polymixin B). Group A antibiotics are the substrate of the efflux pump AcrAB-tolC, whereas group B antibiotics should not efflux out The

strains that were found to be resistant to group A antibiotics and sensitive to group B antibiotics were then selected for further study.

Preparation of Plant Extracts

The parts of plants used for the preparation of extract were washed 2-3 times with tap water, followed by washing with 0.1% $HgCl_2$ to remove the contamination, and then with distilled water. The plant material was shade dried for 4-5 days. Dried plants were grinded into fine powder with the help of a mortar and pestle. Plant powder was stored at 4^0C until further use. Powdered plants were subjected to Soxhlet extraction with methanol as a solvent. The initial concentration of 0.1g/ml (200 ml methanol+25g powder) was prepared. The apparatus was run for 18–24 hours to get a final concentrated slurry. The extract was poured into china dishes. Methanol was evaporated from the extract by incubation at 35-38^{0}C in a water bath. The powder obtained was weighed and stored in sterile tubes at 4^0C until further use [22]. The total yield of different plants was calculated with the help of the following formula:

$$\text{Yield } \% = \frac{\text{Weight of the plant extract obtained in grams after extraction}}{\text{Total weight of plant powder in grams taken for extraction}} \times 100 \quad (1)$$

Efflux Pump Inhibitory (EPI) Activity of Plant Extract

EPI activity of plant extracts was analyzed by using the Berberine potentiating assay as described by Belofsky *et al.* (2006) [23] and the Ethidium Bromide efflux inhibition assay as described by Kamicker *et al.* (2008) [24]. This assay was used as an indicator to identify efflux pump inhibitors present in the plant extracts. The assay was performed with slight modifications. Briefly, overnight cultured *K. pneumoniae* was centrifuged for 2 min at 12000 rpm. To the pellet, 0.5mL of 1M glucose was added, and two different concentrations of plant extract (100 µg/ml and 1000µg/ml) were used. A volume of 170 l of nutrient broth and 5 l of K. pneumoniae culture were loaded into each well of a 96-well microtiter plate, followed by 20L of Berberine (30 g of Berberine dissolved in one ml of DMSO) and 5L of plant extract (15 g of plant extract dissolved in one ml of DMSO). DMSO containing Berberine and bacterial culture was used as a negative control. A well-known EPI, CCCP, was used as a positive control, which was added to culture along with Berberine. Microtiter plates were incubated at 370 C for 24 hours. Then, OD was taken at a wavelength of 595nm in an ELISA plate reader (BioTek). An OD <0.04 is considered to reveal no bacterial growth. No bacterial growth in the presence of Berberine indicates the presence of an MDR inhibitor in the plant extract.

Ethidium bromide efficiently effluxes out and only accumulates in cells in the presence of an efflux pump inhibitor and emits strong fluorescence. The assay was performed in 96 well ELISA plates in duplicate, as described by Kamicker *et al.* [24]. A volume of 170 µL nutrient broth, 5 µL of inoculum, 20 µL EtBr, and 5 µL plant extract was added to each well. The CCCP was used as a positive control, and DMSO was used as a negative control. The fluorescence of the accumulated Ethidium bromide was measured after 30 min with a 5-minute interval at an excitation wavelength of 530 nm and an emission wavelength of 600 nm. The fluorescent ELISA reader (Fluoroskan Ascent, Thermo Fisher Scientific) was used to take the reading.

Phyto-Constituent Analysis of Crude Methanolic Plant Extract

The crude methanolic plant extracts selected by screening were then analyzed for all the major groups of phytocompounds by using standard phytochemical assays according to the previously described procedures [25]. The screening was carried out to determine the presence of active secondary plant metabolites. The plant extracts were screened for the presence of reducing Sugars, Alkaloids, Saponins, Tannins, Flavonoids, Anthraquinones, Phlobatannin, Steroids, Terpenoids, and Cardiac Glycosides.

Synergistic Activity of Plant Extracts

To determine the synergistic activity between antibiotics and plant extracts, the Minimum Inhibitory Concentration (MIC) of antibiotics and plant extracts was carried out first. Then combinations of different concentrations ranging from 1/2X MIC to 8X MIC of each were used. The assay was performed in 96-well ELISA plates. The fixed concentration of plant extract/active compounds was determined to be able to decrease the MIC of resistant antibiotics [26]. The Fractional Inhibitory Concentration (FIC) was determined as FIC index = (MIC of antibiotic in combination/ MIC of antibiotic alone) + (MIC of plant extract in combination/ MIC of plant extract alone). Combinations were classified as synergistic if FIC indices were 1, additive if FIC indices were equal to 1, indifferent if FIC indices were between 1 and 2, and antagonistic if FIC indices were greater than 2.

Thin Layer Chromatography (TLC) of Crude Plant Extract of Terminalia chebula

TLC analysis of a methanolic extract of the plant showed potential synergistic activity (*T. chebula*) was performed by using aluminum coated plates (MERCK) with different combinations of solvents like chloroform, acetone, formic acid, acetic acid, ethanol, methanol, etc. Acetic acid, ethanol, and methanol were used as solvents in a 0.1:5:5 ratio. The spots revealed were visualized by using the iodine chamber along with spray reagents.

Extraction of Bioactive Compound from Terminalia chebula

Bioassay-Guided Fractionation

The isolation of bioactive compounds from *T. cehbula* fruit was done as described by Mahajan and Pai, 2010 [26]. To summarize, two kilograms of dry powdered *T. cehbula* fruits were extracted with methanol. Methanol was evaporated from the extract at 40^0C and dried till the moisture content was 7-8%. A volume of 1.6 liters of alcohol was added and the mixture was refluxed three times at 50 o C while being vacuumed at 225 mm-Hg. The extract was steam distilled, followed by chemical fractions. The 30 g of dry fruit extract was dissolved in 750 ml of HPLC-grade water, followed by the addition of n-Hexane to remove the fat from the extract. Further, the extract was fractioned by chloroform to remove nonpolar compounds that may have been retained on the column. The aqueous layer was finally fractioned with ethyl acetate. The ethyl acetate layer was dried with anhydrous sodium sulphate and evaporated under reduced pressure on the rotary evaporator to yield a residue.

Chromatography of Fruit Extract

On silica gel, column chromatography of fruit extract was performed to extract the compound. A total of six fractions were obtained from *T. chebula* fruit extract and stored for further use. The synergy assay was performed for all six fractions obtained from column chromatography. The fractions showing synergistic activity were then characterized to know LC-MS determined the physical properties of the bioactive compound and molecular weight, and the structure of the bioactive molecule was elucidated by NMR and compared with the data available.

Bioactive Testing

This was carried out to assess the potential of the compound to enhance antimicrobial activity and overcome antibacterial resistance. The three groups are used for the bioactive assessment of bioactive compounds **(Table 1)**.

Table 1. Bioactivity Testing of Bioactive Compound Ethyl Gallate Extracted and Isolated from Methanolic Fruit Extract of *T. Chebula*

Group	Compounds used for bioactivity assay	Concentration of the compound
A	**Ethyl gallate alone**	100 µg/ml
B	**Antibiotics alone**	1. Tetracycline = 30 µg/ml 2. Ciprofloxacin = 5 µg/ml 3. Chloramphenicol = 30 µg/ml
C	**Ethyl gallate + antibiotics**	Ethyl gallate= 100 µg/ml + 1. Tetracycline = 30 µg/ml or 2. Ciprofloxacin = 5 µg/ml or 3. Chloramphenicol = 30 µg/ml

Statistical Analysis

Results obtained were analyzed statistically, and values were expressed as Mean ± SD. Statistical analysis of collected data was also conducted using CRD with three factorial analyses carried out on three factors. For testing the significance of differences between treatments, the least significant difference at the 5% (p 0.05) level was used [27].

Results and Discussion

Antibiotic Susceptibility Assay

In this study, all 40 clinical isolates of *K. pneumoniae* were maintained in *in-vitro* culture, and a drug sensitivity assay of these isolates showed 57.5% (23/40) resistance against Ciprofloxacin and 20% (8/40) against tetracycline and chloramphenicol. Thus, ciprofloxacin resistance was observed to be high among the isolates. Only 5/40 (12.5%) isolates were found multi-drug resistant to Ciprofloxacin, tetracycline, and chloramphenicol. The frequency of MDR isolates was found to be significantly lower among the isolates. Further, an anti-microbial activity assay with group A and group B antibiotics determined the presence of the AcrAB efflux pump in only 2 clinical isolates Group A antibiotic-resistant isolates were used for further EPI study because they were found phenotypically similar to the active efflux pump AcrAB containing *K. pneumoniae*.

Efflux Pump Inhibitory Activity of Plant Extracts

Both assays, Berberine and Ethidium bromide, determined the efflux pump inhibitory activity in methanolic extracts of 8/33 (32%) plants (*Punica granatum, Pterocarpus marsupium* Roxb., *Centella Asiatica, Glycyrrhiza glabra, Rhytididelphous squarrosus, Foeniculum vulgare, Terminalia chebula,* and *Pelargonium graveolens*) by using 1000 µg /ml of dose, whereas EPI activity was not observed in 100 µg /ml **(Figures 1-3)** against MDR *K. pneumoniae* isolates. The phytochemical analysis of a methanolic extract of these eight medicinal plants revealed the presence of phenols, tannins, reducing sugars, saponins, flavonoids, and terpenoids in the methanolic extract.

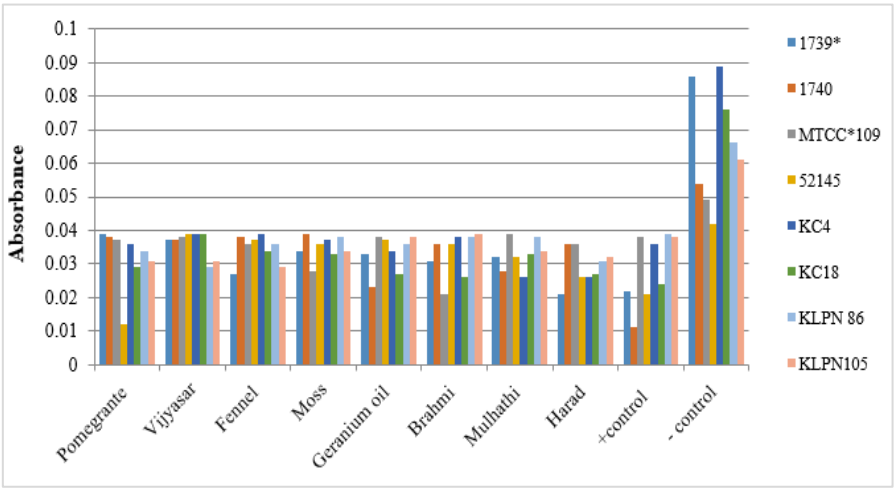

Figure 1. Efflux pump inhibitory activity of methanolic extract of different parts of eight plants at a dose on 1000 µg/mL in Berberine assay (Pomegranate =*P. granatium*, Vijyasar = *P.marsupium* roxb, Brahmi= *C. acsiatica*, Mulhathi = *G. glabra*, Garden Moss = *Rhytididelphus squarrosus*, Fennel = *Foeniculum vulgare*, Harad=*Terminalia chebula*, Germanium oil = *Pelargonium graveolens*)

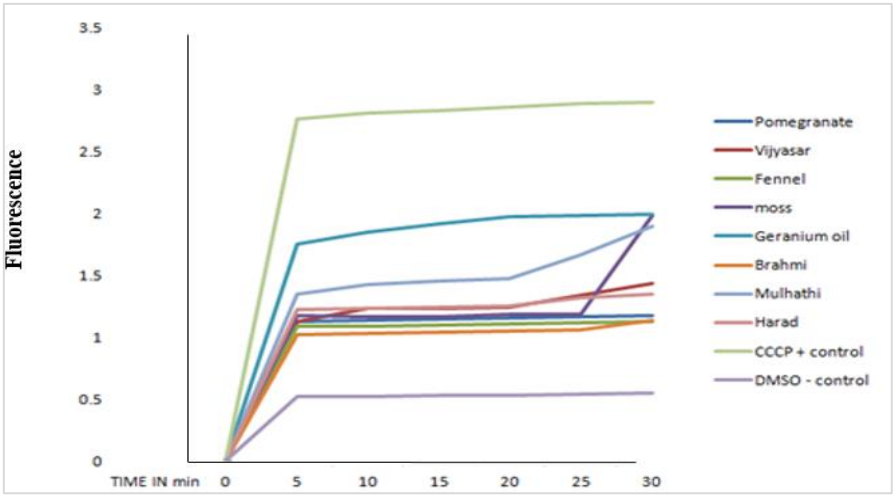

Figure 2. Effect of plant extracts on accumulation of Ethidium bromide at a concentration of 1000 µg/mL by *K. pneumoniae* (Pomegranate =*P. granatium*, Vijyasar = *P.marsupium* roxb, Brahmi= *C. acsiatica*, Mulhathi = *G. glabra*, Garden Moss = *Rhytididelphus squarrosus*, Fennel = *Foeniculum vulgare*, Harad=*Terminalia chebula*, Germanium oil = *Pelargonium graveolens*)

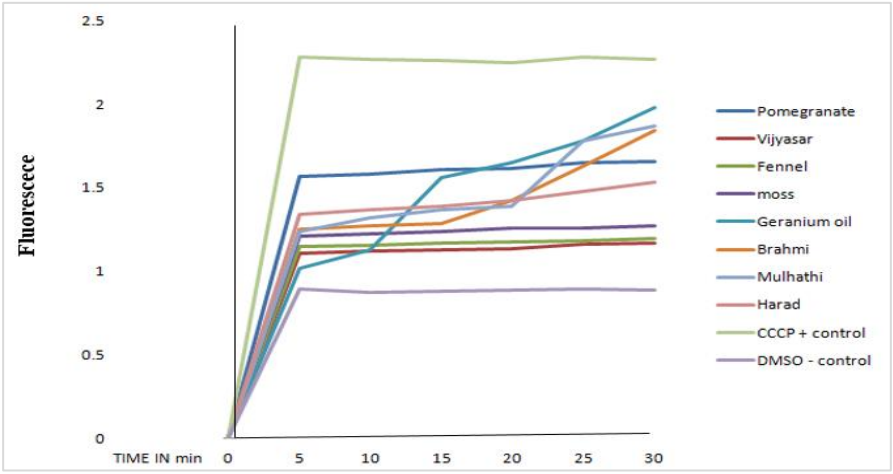

Figure 3. Effect of plant extracts on accumulation of Ethidium bromide at a concentration of 100 µg/mL by *K. pneumoniae* (Pomegranate =*P. granatium*, Vijyasar = *P.marsupium* roxb, Brahmi= *C. acsiatica*, Mulhathi = *G. glabra*, Garden Moss = *Rhytididelphus squarrosus*, Fennel = *Foeniculum vulgare*, Harad=*Terminalia chebula*, Germanium oil = *Pelargonium graveolens*

Synergistic Effect of Plant Extracts with Antibiotics

Of the eight plants, only *T. chebula* has shown a potent synergistic effect with the antibiotics, while other plants have not shown any synergistic effect even after repeated attempts. The 1000 µg/mL concentration of methanolic fruit extract of *T. chebula* has significantly enhanced the activity of resistant group A antibiotics and effectively decreased the minimum inhibitory concentration of antibiotics by up to half). Furthermore, the synergism was determined by the FIC method. By using this method, the MIC of all antibiotics and plant extracts was determined separately, then in combination Potent synergistic activity was observed between methanolic fruit extract of *T. chebula* and group A antibiotics (Ciprofloxacin, Tetracycline, and Chloramphenicol) against MDR strains of *K. pneumoniae* while synergism was not observed against sensitive strains of *K. pneumoniae*. Hence, the observations suggest that the methanolic fruit extract of *T. chebula* contains a bioactive molecule that has EPI activity against MDR strains of *K. pneumoniae. T. chebula* was then selected for further analysis to obtain bioactive compounds containing EPI activity.

Isolation and Characterization of Bioactive Compounds

The TLC of methanolic fruit extract of *T. chebula* also revealed the presence of methyl gallate, ethyl gallate, ellagic acid, and gallic acid (chloroform: methanol, 8:2) with Rf values of 0.66, 0.54, 0.45, and 0.64 respectively. The isolated bioactive compound was observed as a yellow solid crystalline mass obtained by repeated column chromatography and further crystallized from methanol. The molecular weight of the compound was 198.17 m/z, and the melting point was 148-150 ºC with an empirical formula of $C_9H_{10}O_5$. Based on the molecular data obtained, the compound was observed as a gallate derivative and identified as ethyl gallate.

Synergistic Effect of Ethyl Gallate with Antibiotics

Results obtained with different strains of *K. pneumoniae* have shown the significant potential of ethyl gallate as an activity enhancer of the antibiotic composition against not only drug-resistant strains but also drug-sensitive strains **(Table 2)**. Ethyl gallate itself also has antimicrobial activity in comparison to tetracycline and chloramphenicol (MIC = 2 g/ml). However, when ethyl gallate is used in combination with these antibiotics, it enhances the sensitivity of antibiotics drastically (2-40 fold). The FIC analysis has also determined the synergistic activity of Ethyl gallate with Ciprofloxacin, Tetracycline, and Chloramphenicol. In the case of drug-sensitive strains of *K. pneumoniae*, ethyl gallate drastically reduced the concentration of all three antibiotics, *viz.* Ciprofloxacin, Tetracycline, and Chloramphenicol. The decrease ranged from 2 fold to 40 fold, as given in **Table 3**.

Table 2. Synergistic Activity of Ethyl Gallate with Antibiotics Using *K. Pneumonia*

Group	Concentration of antibiotics and Ethyl gallate	*K. pneumonia* strain 52145 (Drug sensitive control) MIC µg/ml	*K. pneumonia* 1740 Knockout strain (Drug resistant) MIC µg/ml
A	**Ethyl gallate alone 1000** µg/ml	**2.00**	**2.00**
B	**Antibiotics alone**		
	1. Tetracycline = 30 µg/ml	0.060	0.125
	2. Ciprofloxacin = 5 µg/ml	2.000	2.000
	3. Chloramphenicol = 30 µg/ml	2.000	2.000
C	**Ethyl gallate + Antibiotics**		
	Ethyl gallate 1000 µg/ml+ Antibiotics		
	1. Tetracycline = 30 µg/ml	0.003	0.003
	2. Ciprofloxacin = 5 µg/ml	0.500	0.500
	3. Chloramphenicol = 30 µg/ml	1.000	0.500

Table 3. Showing the Activity of Ethyl Gallate with Antibiotics (Decrease in Antibiotics Concentration)

Strains	Mic of Antibiotic alone	MIC of Ethyl gallate alone	MIC of Antibiotic + Ethyl gallate	Fold decrease in antibiotics concentration
CIPROFLOXACIN 5 µg/ml				
Sensitive strain*	0.060	2.00	0.003	20
Resistant strain**	0.125	2.00	0.003	40
TETRACYCLINE 30 µg/ml				
Sensitive strain*	2.00	2.00	0.500	4
Resistant strain**	2.00	2.00	0.500	4
CHLORAMPHENICOL 30 µg/ml				
Sensitive strain*	2.00	2.00	1.000	2
Resistant strain**	2.00	2.00	0.500	4

**K. pneumonia* strain 52145 (Drug Sensitive)
***K. pneumonia* strain 1740 (Drug-resistant)

The study suggested ethyl gallate acts as a potent EPI and enhances the activity of resistant antibiotics by several folds, and the addition of it drastically reduces the dose of antibiotic compositions and makes the antibiotics effective against even drug-resistant bacteria.

The spread of antibiotic-resistant strains of bacteria is a serious public health problem and is an alarming issue. If the effectiveness of antibiotics is lost, the ability to treat bacterial infections will be difficult. As more strains of bacteria become resistant to antibiotics, the drug of choice has become increasingly limited and more expensive, which may affect the economies of developing countries [28]. *K. pneumoniae*, a gram-negative microorganism, is a leading cause of morbidity and mortality in developing countries due to increasing multidrug resistance. The World Health Organization has designated *K. pneumoniae* as a primary pathogen of concern for which next-generation antibiotics are urgently needed. Antibiotics that were once effective against *K. pneumoniae*, such as aminoglycosides, nitrofurantoin-lactams, tigecycline, and colistin quinolones [29, 30] are now generally ineffective [14, 31]. One hypothesis for this could be the increased expression of efflux pumps.

The drugs are effluxes from the bacterial cells, reducing the lethal concentration of antibiotics from the cell cytoplasm. Most Gram-negative bacteria acquire multiple families of efflux pumps that decrease the intracellular concentrations of antibiotics, leading to inherent or acquired resistance against different groups of antibiotics [6-8]. There are two major efflux pump families, *viz*., RND and MFS, which are found in gram-negative bacteria. Members of the RND family have been reported to be major contributors to multidrug resistance among efflux pumps [32, 33]. The RND family of OqxB efflux pumps has also been identified as a significant contributor to antimicrobial resistance in *E. cloi* and *K. pneumonia*. The role of intracellular antibiotic concentration in *K. pneumoniae* and developing MDR strains by the AcrAB-TolC pump is well documented [34, 35]. Hence, the discovery of a potent efflux pump inhibitor is urgently needed. We discovered a plant-derived EPI that is significantly ineffective of the AcrAB-TolC pump and effectively capable of increasing the sensitivity of resistant antibiotics in MDR K. pneumonia in this study.

Forty clinical isolates of *K. pneumoniae* were studied. Antibiotic resistance patterns were determined among the isolates, and isolates found resistant to two or more structurally distinct classes of antibiotics were included in this study. In the present study, 57.5% of isolates were observed to be resistant to Ciprofloxacin and 20% to Tetracycline and chloramphenicol. Ali *et al.* (2010) [36] observed very significant resistance in *K. pneumoniae* against ciprofloxacin from Pakistan, which is consistent with our findings. Multidrug resistance is also reported in *A. baoumannii, E. coli, P. aeruginosa,* and *S. aureous* in similar ways [37, 38]. However AcrAB-TolC efflux pump meidiated MDR has also been reported in *E. coli* [39]. As a result of this research, it appears that MDR patterns in gram-negative bacteria have spread widely over the world. In accordance with the present study, RND mediated multidrug resistance has been reported in *vibrio* spp. isolated from the mummichog fish gut. [40].

In this study, we focused on the EPI activity of bioactive molecules derived from plants. The well-known EPIs Carbonyl Cyanide m-Chlorophenyl hydrazone was used as a positive control. We observed 24.25% of plants showing EPI activity and also enhanced the activity of the antibiotic composition. *T. chebula* fruit extract has shown productive synergistic activity with antibiotic compositions *viz.* Ciprofloxacin, Tetracycline, and Chloramphenicol and effective control of the growth of multidrug-resistant isolates of *K. pneumoniae*. It has been reported that the *T. chebula* fruit extract has a resistance-modifying potential against multidrug-resistant pathogens [41], which reinforces our findings. Similarly, Aqil *et al.* (2005) [42] reported that the *T. chebula* fruit extract enhances the activity of tetracycline against resistant strains of *S. aureus.* Khalid *et al*. (2017) [43] also discovered that the bark of *T. aphylla L.* and the stem of *A. Arabica L*. has antibacterial properties against multidrug-resistant dental biofilm-producing bacterial strains. Similar to our findings, *Punica granatum* peel extracts showed inhibitory efficacy against clinical MDR isolates in HPLC fractions [44].

The EPI activity of *T. chebula* has also been reported against MDR strains of *E. coli* [41]. Also, it was demonstrated that *T. chebula* enhanced the effectiveness of Novobiocin antibiotics against resistant strains of *A. Baumannii* [45]. In the present study, *T. chebula* was discovered to have significant activity and reduced the MIC of all three antibiotics (ciprofloxacin, tetracycline, and chloramphenicol), with further synergism being established using the FIC method. The discovery of Ethyl gallate, a bioactive molecule derived from *T. chebula* methanolic extract, as a source of potential efflux pump inhibitor that effectively increases the sensitivity of commonly used antibiotics against multi-drug resistant Gram-negative bacteria *K. pneumoniae* opened the door to new avenues in controlling emerging drug resistance and suggested that Ethyl gallate (Indian Patent, International Patent, and U.S. Patent) may be used as a source of

A large number of MDR strains is required to make any conclusion, whereas, in the present study, the number of strains was less. However, the controls used in this study were standard strains that are genetically confirmed for the activity of efflux pumps in bacteria. Ethyl gallate, based on our experimental evidence, could be a potent EPI [46]. As previously discovered, EPIs were mostly against gram-positive bacteria, so these new findings may prove very helpful in demolishing the efflux pumps in gram-negative bacteria as well.

Conclusion

The methanolic extract of *T. chebula* may be used as a source of putative EPI against Gram-negative bacteria and may be tried for clinical use. The emergence of multi-drug resistance in Gram-negative bacteria is a significant challenge, and the control of these pathogenic bacteria is complicated by existing control measures. Plants are the major sources of bioactive molecules that can control pathogenic organisms. Our study based on the small number of strains showed that Ethyl gallate may be used

as a source of putative efflux pump inhibitor in combination with antibiotics for the treatment of multidrug-resistant strains of *K. pneumoniae*. The controls used in this study were standard strains that were genetically confirmed for the activity of efflux pumps in bacteria. The findings of our study suggest that Ethyl gallate could be a potent EPI.

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Conflict of interest: None

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