SVOA Microbiology

ISSN: 2634-534X

ScienceVolks Open Access

Research Article

Ethyl Gallate: A Potent Inhibitor of MexAB-OprM Efflux Pump in Multidrug-Resistant *Pseudomonas aeruginosa*

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DOI: https://doi.org/10.58624/SVOAMB.2024.05.054

***Received:** August 16, 2024* ***Published:** September 27, 2024*

Abstract

Drug resistance in *P. aeruginosa* has been mediated by several mechanism, out of them efflux pump mediated resistance is one of the most important mechanisms of drug resistance. MexAB-oprM efflux pump capable of recognizing and expelling a variety of structurally unrelated compounds from the bacterial cell confers resistance to a wide range of antibiotics in *P. aeruginosa*. The aim of the present study was to screen medicinal plants used in Indian traditional medicine to find some potent compound(s) capable of inhibiting the MexAB-oprM pump in *P. aeruginosa* and also study the synergistic effect of characterized efflux pump inhibitor(s) with resistant antibiotics in Multi-Drug Resistant (MDR) strains of *P.aeruginosa*. A total of 100 clinical isolates, four knockouts and one MTCC-741 standard strain of *P. aeruginosa* were used in this study. All the 100 clinical isolates were processed for antibiotic susceptibility assay and EtBr Agar Cartwheel assay for determination of MDR phenotype. A total of 40 plants were screened for the presence of compounds with efflux pump inhibitory activity. The plants showing EPI activity were further explored for their synergistic effect with three different antibiotics. Ten plant extracts have shown considerable EPI activity and out of the 10 active extracts only one methanolic extract of *Terminalia chebula* fruits has shown synergistic activity with group A (ciprofloxacin, tetracycline and chloramphenicol). The fractionation and purification of *T. chebula* fruit extract provided ethyl gallate which have shown synergistic activity along with group A antibiotics as well as significant EPI activity. The results of the present study conclude that ethyl gallate works as a potent EPI against overexpressing MexAB-oprM efflux pump in *P. aeruginosa* and may be used along with the resistant group A antibiotics against Multi-Drug-Resistant *P. aeruginosa*.

***Keywords:** Pseudomonas aeruginosa, Multi-Drug Resistance, MexAB-oprM pump, Efflux pump inhibitors (EPI), Terminalia chebula.*

1. Introduction

Drug resistance in microbiological pathogens has become a major public health concern, restricting the use of previously prescribed, easily accessible, and affordable antibiotics. The non-fermentative, aerobic, rod-shaped bacteria *Pseudomonas aeruginosa* causes several potentially fatal human problems, including pneumonia, endocarditis, and skin and soft tissue infections. The most prevalent kind of pseudomonad, *P. aeruginosa*, is frequently linked to infections in people and is also a common cause of nosocomial infections (Warren et al., 2000, Banerjee et al., 2000).

By several methods, *P. aeruginosa* can readily develop resistance to all traditional anti-pseudomonal medicines (Abbas et al; 2013). Enzymatic inactivation (Abdi-Ali A et al; 2006), drug target(s) alteration (Adwan MG et al; 2008), and changes in membrane permeability or overexpression of efflux pumps (Ahmed SM et al; 2012) to reduce intracellular drug concentration are the major processes. Membrane-associated active transporters known as efflux pumps provide a self-defence mechanism by actively removing medications from cells. This lowers the concentration of the antibiotic in the cell and eventually makes the bacteria resistant to the medication (Akama H et al; 2004). The five super families that comprise bacterial antibiotic efflux pumps are the Multidrug and Toxic Compound Extrusion (MATE) superfamily, Resistance Nodulation Division (RND) superfamily, ATP Binding Cassette (ABC) transporters, Major Facilitator Superfamily (MFS), and Small Multidrug Resistance (SMR) superfamily (Pages et al., 2005).

P. aeruginosa exhibits significant multidrug resistance due in part to these many efflux pumps (Schweizer, 2003). The single specific efflux pump in *P. aeruginosa*, MexAB-OprM, which is a member of the RND superfamily, is expressed constitutively in the bacterial cells where it adds to intrinsic resistance to a variety of antibacterial agents, such as tetracycline, chloramphenicol, β-lactams, and fluoroquinolones (Li et al., 1998). Efflux pumps are legitimate targets for antibacterial drugs, and finding strong inhibitors of efflux pumps is a promising strategy that can make resistant strains susceptible to antibacterial treatments again [Akiyama H et al; 2001, Akram M et al; 2007].

Numerous secondary metabolites that are produced by plants have been found to have medicinal potential. Of the world's 2,500,00 species of higher plants, only 5–10% have undergone chemical research (Tshibangu et al., 2001). According to reports in the literature, crude extracts and chemicals produced from plant sources have been used against almost every known pathogen, including yeasts, fungus, bacteria, and parasites. Because of their synergistic effects, compounds derived from plants have also been used in conjunction with antibiotics to develop therapies for infections caused by bacterial species (Coutinho et al.,2009). These compounds may enhance the activity of antibiotics in combination. Along with known anti-Pseudomonal antibiotics against *P. aeruginosa*, the goal of the current study was to screen a few plants used in Indian traditional medicine for efflux pump inhibitory and synergistic activity. Additionally, the compounds from the active extracts were to be further isolated, purified, and characterised, and their EPI and synergistic activities were to be confirmed.

2. Materials and Methods

2.1. Collection of clinical and knockouts strains of *Pseudomonas aeruginosa*

In all, 100 *P. aeruginosa* clinical isolates were gathered from the microbiology department of Gian Sagar Medical College in Patiala, Punjab, India. The Department of Microbiology and Molecular Medicine at the University of Geneva in Genève, Switzerland provided the cultures of the knockout strains of *P. aeruginosa*, which include one wild type of strain, R3-(PAO1), one hyper susceptible strain, R1-(TETR-T), and two strains that overexpress the MexAB-oprM efflux pump, R2-(TETR) and R4-(PT629). The Institutional Ethics Committee provided an ethical clearance certificate (reference number: SUIEC/13/26).

2.2. Cultivation and characterization of *P.aeruginosa*

Nutrient agar plates were infected with each of the 100 clinical isolates of *P. aeruginosa*, and the plates were then incubated for 24 hours at 37°C. At -80°C, all the cultures were kept in stocks of 15% and 40% glycerol. *P. aeruginosa* was characterised by growing on selective media (Simmon's Citrate Agar), Gramme staining, and biochemical analysis.

2.3. Antibiotic sensitivity assay

For the antibiotic sensitivity test, group A antibiotics that are refluxed out by *P. aeruginosa's* MexAB-oprM efflux pump and group B antibiotics that are not effluxed out by MexAB-oprM efflux pump of *P. aeruginosa* were chosen (Table 2.1). MDR phenotypes were screened using the traditional disc diffusion assay. For additional research, strains that were both sensitive to group B antibiotics and resistant to group A antibiotics were chosen.

Table 2.1: Group A and B antibiotics used as substrate and non-substrates for MexAB-OprM efflux pump of *P.aeruginosa* respectively.

SERIAL NO:	GROUP-A (SUBTRATES OF MexAB- OprM EF-FLUX PUMP)	GROUP-B (NON-SUBTRATES OF MexAB- OprM EFFLUX PUMP)
1.	Chloramphenicol	Meropenem
2.	Ciprofloxacin	Cefepime
3.	Tetracycline	Imipenem
4.	β-lactams	Cefpirome
5.	Novobiocin	Carbenicilli and sulbenicillin
6.	Trimethoprim	

2.4. Agar Cartwheel assay for the detection of efflux pumps

The EtBr-Agar Cartwheel (EtBrCW) method was used to assess the presence of efflux activity in confirmed MDR strains with brief modifications (Martins *et al*., 2013 Costa *et al*., 2013). All the strains of *P. aeruginosa* were grown in nutrient broth instead of Tryptic Soya Broth and incubated at 37 degrees for 24 hours. Then culture was inoculated by swabbing on Tryptic Soy Agar containing Ethidium Bromide. Three different concentrations of EtBr were used i.e., 2µ g/mL, 1µg/mL and 0.5µg/mL, these concentrations were lower than the MIC of EtBr. Plates were incubated for 16 hours at 37 degrees and then observed under UV light (Gel Doc-It2 310 Imager). The effect of temperature was also recorded by incubation at 37 degrees and 4 degrees for 16 hours. The fluorescence at each temperature was compared to that evident after the first incubation at 37 degrees.

2.5. Screening of methanolic plant extracts for Efflux Pump Inhibitory (EPI) activity

2.5.1. Collection of plants

All the twenty (20) authenticated plants were collected from Y. S. Parmar University of Horticulture and Forestry Nauni, Solan, Himachal Pradesh, India and Arya Vastu Bhandar, Dehradun, Uttrakhand, India.

2.5.2. Preparation of Plant Extracts

The parts of plants used for the preparation of extracts were washed with tap water 2 to 3 times and then washed with 0.1% Hg Cl2 to remove the contamination, followed by washing with distilled water. Cleaned plant parts were shade dried for 4 to 5 days. The dried plant parts were ground to yield a coarse powder with the help of mortar and pestle. The coarse powders were then subjected to Soxhlet extraction with methanol for 18-24 hours. The extracts were then dried by incubation at 35-38°C. The dried extracts were stored in airtight bottles at 4°C.

2.5.3. Berberine potentiation assay

Berberine is a substrate for efflux pumps and well-known antimicrobial agent and effluxes out by resistant bacteria through efflux pumps. Berberine assay was used as a marker to identify efflux pump inhibitors present in plant extracts. Stermitz *et al*., (2000) and Belofsky *et al*., (2006). Cultures of *P. aeruginosa* were incubated at 37ºC for 24 hours. The culture was centrifuged for 2 minutes at 12,000 rpm and 0.5 ml, 1 M glucose was added into the culture which was used for the berberine potentiation assay. The assay was performed in 96 well microtiter plate with two different concentrations for each plant extract *i.e.* 100µg/ml and 1000µg/ml. 175µl of *P. aeruginosa* culture was poured into each well followed by addition of 20µl Berberine (30µg berberine dissolved in 1 ml dimethyl sulfoxide (DMSO) and 5µl of plant extract (15 µg plant extract dissolved in 1ml DMSO). DMSO (20 µl) without plant extract was used as negative control along with the addition of berberine and culture. For positive control, CCCP (a well-known EPI) was added into the culture along with berberine. Plates were incubated at 37°C for 24 hours. Then Optical Density was taken on 595nmin an ELISA plate reader (Bio-Tek). No bacterial growth in the presence of Berberine indicates the presence of an EPI inhibitor in plant extract.

2.5.4 Ethidium Bromide Efflux Inhibition Assay (Kamicker et al.,2008)

Ethidium bromide is efficiently effluxes out and will only accumulate in cells in the presence of an efflux pump inhibitor and emits strong fluorescence. Assay was performed in 96 well ELISA plate in duplicate. For inoculum, a loop of *P. aeruginosa* culture was suspended into 10ml Luria broth and inoculated at37°C. The turbidity of the solution was adjusted according to 0.5 Mc-Farland standard. In each well, 175 µl inoculum, 20 µl EtBr and 5 µl plant extract was added. The known EPI, 20µL CCCP was used as a positive control. 20 µl of DMSO was used as a negative control. Then fluorescence of the accumulated ethidium bromide was measured for 30 minutes with an interval of 5 minutes at excitation wavelength of 530 nm and emission wavelength of 600 nm. Reading was taken in the Fluorescent ELISA reader (Flourochrome, Thermo Fisher Scientific).

2.6 Effect of plant extracts in lowering the MIC of antibiotics

Firstly, MIC of antibiotics and plant extract was determined. To determine the combined effect of antibiotic and plant extract, combinations of different concentrations ranging from 1/2×MIC to 8×MIC of each was used. This assay was performed in 96 well ELISA plate. By this assay a fixed concentration of active compound was determined which decreased the MIC of antibiotic. Following formula was used for the determination of FIC:

FIC index= (MIC of antibiotic in combination/ MIC of antibiotic alone) + (MIC of plant extract in combination/ MIC of plant extract alone).

Combinations are classified as: If the FIC value indices <1, the results will be synergistic, and if FIC value indices =1 the results will be Additive, if FIC value indices b/w 1 & 2 the results will be Indifferent and if FIC indices > 2 the results will be antagonistic.

2.7. Thin Layer Chromatography (TLC) of active plant extract

TLC analysis of methanolic extract of *T. chebula* was also done by using different solvent combinations. Thin layer chromatography was done by using aluminium coated TLC plates (MERCK). The solvent system having acetic acid, ethanol and methanol was used in a ratio of 50µl : 2.5ml : 2.5ml. The spots revealed were visualized using iodine chamber along with spray reagents.

2.8. Isolation of bioactive compound(s) from *Terminalia chebula*

2.8.1. Bioassay guided fractionation

The *T. chebula* methanolic extract (30g) was dissolved in 750 ml of HPLC grade water followed by addition of n-Hexane to de-fat the extract. Then the extract was fractioned by chloroform to remove highly non-polar compounds those may retain in the column. The aqueous layer was then finally fractioned with ethyl acetate. Ethyl acetate layer was dried with anhydrous sodium sulphate and evaporated under reduced pressure by using rotary evaporator to yield concentrated slurry.

2.8.2. Chromatographic separation of the Ethyl acetate fraction

45g of silica gel (60-120 mesh) was activated previously by heating in a hot air oven at 110±5°C for 1h. A 50 cm long glass column fitted with a stopper was filled with 45 g of activated silica gel. The ethyl acetate fraction was subjected to silica gel chromatography eluted with methylene chloride and increasing proportion of methanol from CH2Cl2-MeOH; 10:0 to 5:5 to provide six fractions.

2.9. Synergistic activity of all fractions obtained from chromatography

Synergistic assay was performed for all the fractions obtained from chromatography as described earlier.

2.10. Characterization of the isolated compound

2.10.1. Physical parameters

Color, Melting Point and Solubility of the newly isolated natural compound were determined. Melting point was determined using Thiele's melting point apparatus.

2.10.2. Characterization by spectroscopic methods

Characterization of the isolated compound was done by LC-MS, 1H-NMR, 2-DNMR and13C-NMR.Mass spectrum was recorded on Model Q-ToF Micro (Waters) spectrometer.1H-NMR,2-DNMRand13C-NMR spectral data were recorded in Bruker Avance II 400MHz NMR spectrometer available at SAIF Punjab University Chandigarh.

2.11. EPI and synergistic activity of purified and characterized compound

The purified compound was further evaluated for its synergistic and EPI activity using the previously mentioned protocols.

2.12. Statistical analysis

Results obtained were analysed statistically and values were expressed as Mean ± SD. Statistical analysis of collected data was also carried out using CRD three factorial analysis. The least significant difference at 5% level was used for testing the significant differences among treatments (Nigam *et al*., 1979).

3. Results

3.1. Collection of clinical isolates of *Pseudomonas aeruginosa*

A total of one hundred clinical isolates of *P. aeruginosa,* four knockout strains of *P. aeruginosa* (Table-3.1). Four strains of *P. aeruginosa* including, one wild type and three MexAB-oprM efflux pump knockouts/overexpressing strains were procured from Dr. Thilo Kohler, University of Geneva, and department of Microbiology and molecular medicine Geneve, Switzerland. One standard sensitive strain of *P. aeruginosa* MTCC-741 were collected for the study was obtained from IMTECH, Chandigarh, India, used as a control.

Table 4.1: Collection of different Knockouts strains of *P. aeruginosa* (n=4).

Strain no.	Strain name	Strain description	Sensitivity
R1	TETR-T	(Derivative of TETR mutated in oprM by insertion of Hg cassette, hypersusceptible strain)-Negative control	S
R2	TETR	(PAO1 derivative, overexpressing strain of MexAB-oprM, resistant strain)-Positive control	R
R3	PAO1	(Wild type strain of MexAB-oprM pump, resistant strain)-Positive control	R
R4	PT629	(PAO1 nalB derivative, 2 bp deletion in MexR, overexpresses MexAB-oprM, resistant strain)-Positive control	R

3.2. *In vitro* Cultivation of *P. aeruginosa* clinicalisolates

The culture of all four knockouts strains of *P. aeruginosa* one wild type strain namely R3-(PAO1) of *P. aeruginosa*, one hyper susceptible strain *i.e.* MexAB-oprM efflux pump knockout strain namely R1-(TETR-T) and two strains of *P.aeruginosa* that overexpresses MexAB-oprM efflux pump namely R2-(TETR) and R4-(PT629), One hundred clinical isolates of *P. aeruginosa* and One standard MTCC-741 sensitive strain of *P. aeruginosa* was cultivated in nutrient agar. Opaque, irregular, slimy with earthy smell appeared on nutrient agar plate.

3.3. Characterization of *P. aeruginosa*

Bacilli of *P. aeruginosa* were seen under the microscope characteristics. The green pigments turned into blue, which is a characteristic feature of *P. aeruginosa* (Figure 4.4).

Fig. 4.4: *Culturing of P. aeruginosa on Simmon Citrate Agar media showing characteristic blue pigments.*

3.4. Antibiotic Susceptibility Testing

Antibiotic susceptibility assay was performed for all one hundred clinical isolates. Out of one hundred clinical isolates six (6%) were found MDR. The frequency of MDR isolates was found to be significantly low among the isolates. Antimicrobial activity of group A and group B antibiotics were performed for six MDR isolates. Out of six, three isolates were found resistant to group A and sensitive to group B (Table 4.4). These three isolates were selected for further study because they were found phenotypically like active efflux pump MexAB-oprM containing *P.aeruginosa*.

Table 4.4: Antibiotic susceptibility assay of MDR *P.aeruginosa* isolates for group A and Group B antibiotics (n=5).

S.No	MDR Strains of *P.aeruginosa* (n=6)	Group A						Group B				
		C	CIP	TE	TR	NV	P	IPM	CPM	CFP	MRP	CBS
1	Ps-3	R	R	R	R	R	R	S	S	S	S	S
2	Ps-T10	R	R	R	R	R	R	S	S	S	S	S
3	Ps-11	R	R	R	R	R	R	S	S	S	S	S
4	Ps-24	R	R	R	R	R	R	S	S	S	S	S
5	Ps-40	R	R	R	R	R	R	S	S	S	S	S
6	Ps-92	R	R	R	R	R	R	S	S	S	S	S

C-Chloramphenicol, CIP-Ciprofloxacin, TE-Tetracycline, TR-Trimethoprim, NV-Novobiocin, P-Penicillin (β-lactams), IMP-Imipenem, CPM-Cefepime, CFP-Cefpirome, MRP-Meropenem, CBS-Carbenicilli and sulbenicillin.

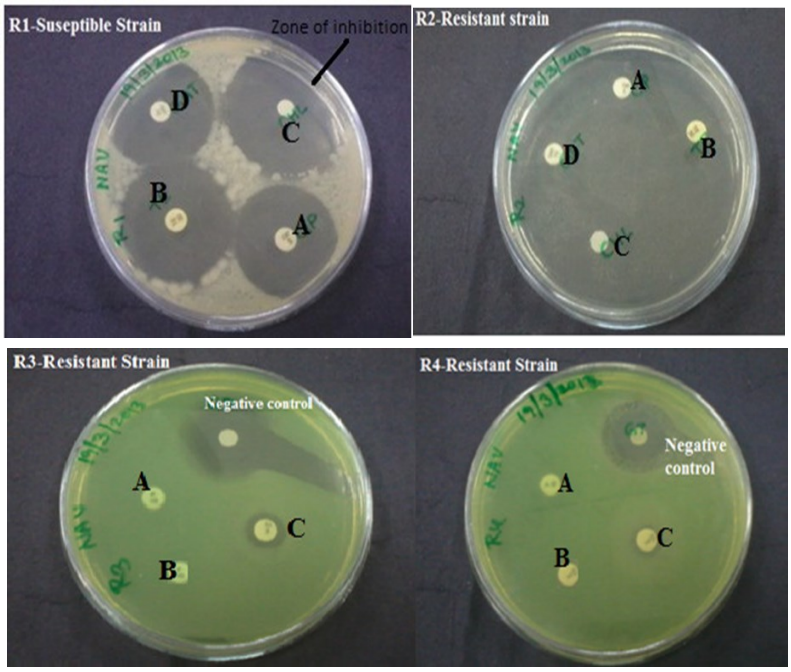


Fig 4.1: *Antibiotic susceptibility using disc diffusion assay (A) P. aeruginosa (R1- Susceptible isolate), (B) P. aeruginosa (R2-Resistant isolate), (C) P. aeruginosa (R3- Resistant isolate), (D) P. aeruginosa (R4-Resistant isolate) and one negative control.*

A*=Ciprofloxacin, B*=Tetracycline, C*=Chloramphenicol, D*=Trimethoprim

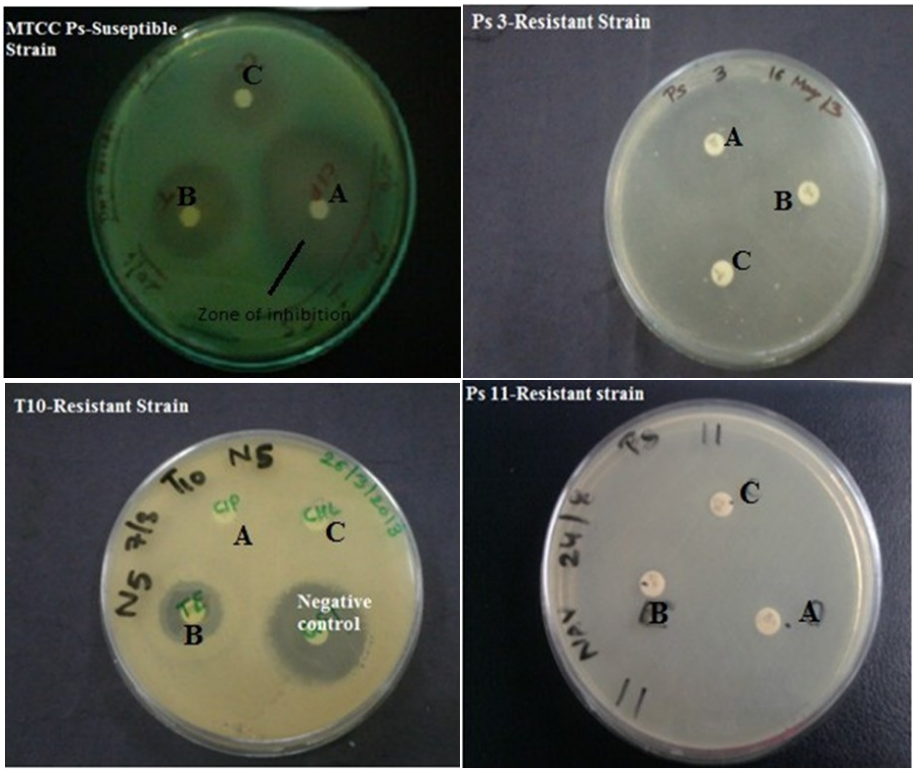


Fig 4.2: *Antibiotic susceptibility using disc diffusion assay (A) P. aeruginosa (MTCC- 471-Reference strain) Susceptible strain, (B) P. aeruginosa (Ps3- Clinical isolate) Resistant strain, (C) P. aeruginosa (Ps T10- Clinical isolate) Resistant strain, (D) P. aeruginosa (Ps11-Clinical isolate) Resistant* strain and one negative control.

A*=Ciprofloxacin, B*=Tetracycline, C*=Chloramphenicol, D*=Trimethoprim

3.5. Agar Cartwheel Assay

EtBr Agar Cartwheel assay was performed to confirm the presence of efflux pumps for all three MDR clinical isolates resistant to group A and for all five specific strains including one wild type (R3-PAO1), three knockout/overexpressing (knockout strainnamelyR1-TETR-T), (overexpressing MexAB-oprM efflux pump namely R2-TETR and R4-PT629) and one standard strain of MTCC-471(Sensitive strain) of *P. aeruginosa*. Both the group-A MDR clinical isolates as well as four MexAB-oprM efflux pump overexpressed strains of *P. aeruginosa* have not shown the EtBr fluorescence hence it confirms the presence of efflux pump because the EtBr effluxes out from the bacteria while EtBr fluorescence was observed in one efflux pump knockout strains and in MTCC-471 standard strain (Figure 4.5).

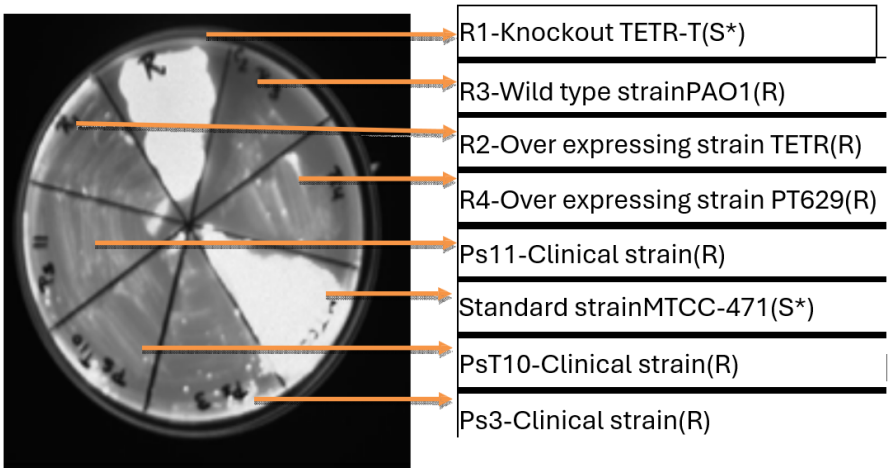


Fig 4.5. *Ethidium bromide fluorescence detected in two sensitive strains of P.aeruginosa. The resistance strains showing the absence of EtBr fluorescence confirming the presence of Efflux pump.*

3.6. Screening of methanolic plant extracts for Efflux Pump Inhibitory (EPI) activity

Two assays were performed to evaluate the efflux pump inhibitory activity of plant extracts.

3.6.1. Berberine Potentiation Assay

Berberine works as an efflux pump substrate and inhibits growth of bacteria in the presence of EPI. Therefore, berberine is used as a marker to find out the presence of an EPI in plant extracts. Berberine assay was performed for methanolic extracts of 40 plants against three MDR clinical isolates and all five specific strains including one wild type (R3-PAO1), three knockout/overexpressing (knockout strain namely R1-TETR-T), (overexpressing MexAB-oprM efflux pump namely R2-TETR and R4-PT629) and one standard strain of MTCC-471 (Sensitive strain) of *P. aeruginosa*. Two different concentrations of berberine were used, 100µg/ml and 1000 µg/ml to perform the sensitivity assay. The efflux pump inhibitory activity was observed in 10 methanolic extracts while 30 methanolic extracts have not shown any efflux pump inhibitory activity (Table 4.8). The 1000 µg/ml of concentration was observed potent and effective concentration while 100 µg/ml concentration of all plant extract have shown less EPI activity for *P. aeruginosa* (Fig. 4.7 and 4.8).

Table 4.8. Detection of EPI activity in methanolic plant extracts by using berberine as a marker for different MDR strains of *P. aeruginosa*.

S. no	English name	Botanical name	Part used	EPI activity
1.	Blackmyrobalm	*Terminalia chebula*	Dry fruits	Present
2.	Currytree	*Murraya koenigii*	Leaves	Present
3.	Ginger	*Zingiber officinale*	Rhizome	Present
4.	Hilllemon	*Citrus pseudolimon*	Leaves	Present
5.	Brahmi	*Centella asiatica*	Leaves	Present
6.	Jambolan	*Syzygium cumini*	Leaves, stembark	Present
7.	Pomegranate	*Punica granatum*	Fruits	Present
8.	Sweetwood	*Glycyrrhiza glabra*	Root	Present
9.	Turmeric	*Curcuma longa*	Root	Present
10.	Geranium	*Pelargonium graveolens*	Leaves-oil	Present

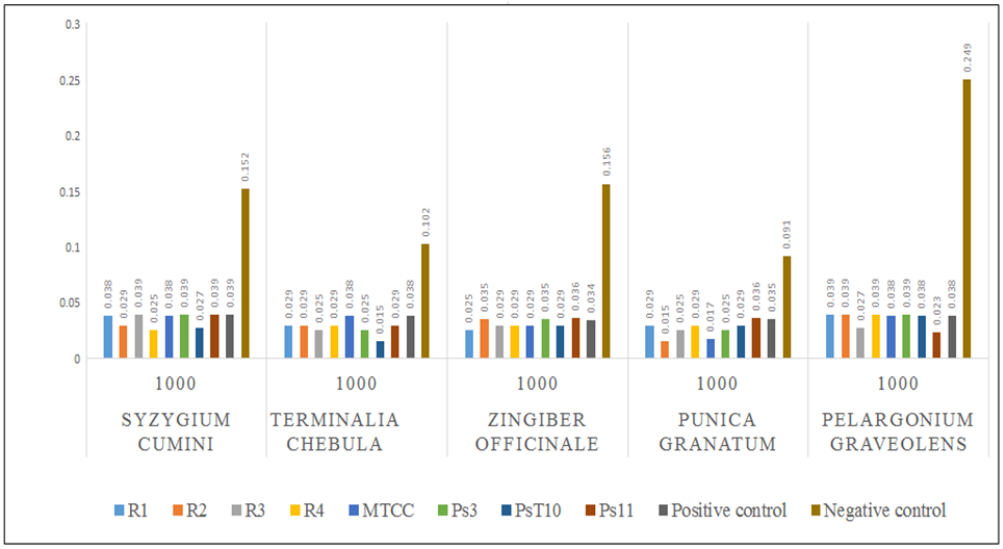


Figure 4.7. *Absorbance shown by S. cumini,T. chebula, Z. officinale, P. granatum and P. graveolens at a concentration of 1000µg/ml by P. aeruginosa strains.*

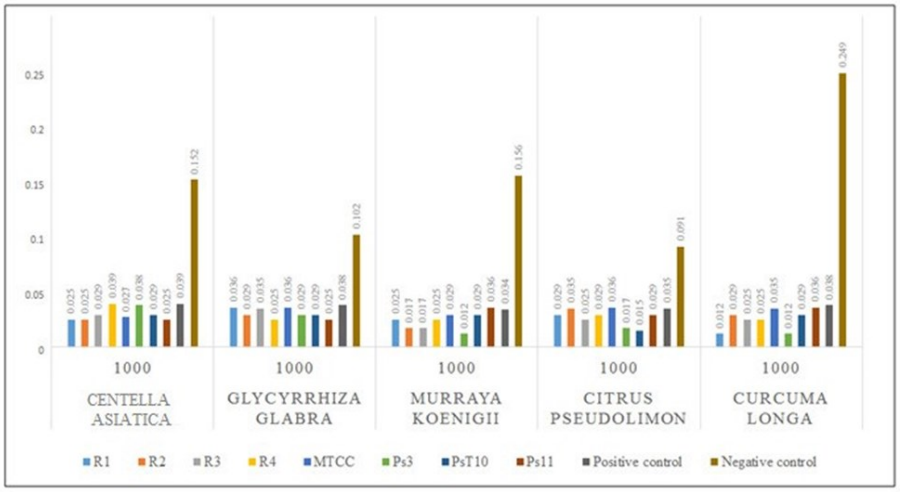


Figure 4.8. *Absorbance shown by C. asiatica, G. glabra, M. koenigii, C. pseudolimon and C. longa at a concentration of 1000 µg/ml by P. aeruginosa*

3.6.2. Ethidium Bromide Assay for the detection of EPI activity in methanolic plant extracts

Ethidium Bromide is a florescent dye and gets accumulated in MDR efflux pump containing bacteria with EPI and shows fluorescence after accumulation. Hence, it is used as a marker for the detection of EPI in plant extracts. EtBr assay was performed for methanolic extracts of 40 plants against three MDR clinical isolates and five standard strains (1 wild type, 2 MexAB-oprM over expressive strains, 1 mutated knockout strain of MexAB- oprM and 1 MTCC-471) of *P. aeruginosa*. The methanolic extracts of only10/40 (25%) plant extracts have shown the efflux pump inhibitory activity. Hence the observations of EtBr assay were found like berberine assay as shown in Table 4.10. and confirmed the Berberine assay.

3.7. Synergistic effect of plant extracts with antibiotics

All the ten plants (*Syzygium cumini, Terminalia chebula, Zingiber officinale, Punica granatum, Pelargonium graveolens, Centellaasiatica, Glycyrrhiza glabra, Murraya koenigii, Citrus pseudolimon* and *Curcuma longa*) showing EPI activity with berberine and ethidium bromide assay were further explored for their synergistic activity with commonly used drug ciprofloxacin, tetracycline and chloramphenicol. Out of 10 plants two methanolic extracts of *T. chebula* and *S. cumini* have shown potent synergistic activity with the antibiotics while other plants have not shown any synergistic effect even after repeated attempts. The 1000 µg/ml concentration of *T. chebula* and *S. cumini* was observed effective with above mentioned antibiotics and effectively decreased the minimum inhibitory concentration of antibiotics (Table 4.9 and Table 4.11).

3.8. Fractional Inhibitory Concentration (FIC)

The synergism, antagonism and indifferent activity between the methanolic plant extracts and three antibiotics (Ciprofloxacin, chloramphenicol and tetracycline) was determined by Fractional Inhibitory Concentration method. By using this method, MIC of all antibiotics and plant extract was determined separately than in combination (Table 4.10 and Table 4.12). Methanolic fruit extract of *T. chebula* and methanolic leaf extract of *S. cumini* has shown potent synergistic activity with all the three antibiotics (ciprofloxacin, tetracycline and chloramphenicol) for MDR strains of *P. aeruginosa* while the sensitive strains have not shown any synergistic effects with methanolic fruit extract of *T. chebula* and leaf extract of *S. cumini*. Hence, the observation suggests that the methanolic extract of *T. chebula* and *S. cumini* contains bioactive molecule(s) which may have EPI activity against MDR strains of *P.aeruginosa*.

3.9. Identification of the compounds present in *Terminalia chebula* fruit extract

3.9.1. TLC analysis of methanolic fruit extract of *T. chebula*

The TLC of methanolic fruit extract of *T. chebula* was performed to know the phyto-constituents present in the extract. Methyl gallate, Ethyl gallate, Ellagic acid and Gallic acid with Rf values 0.64, 0.66, 0.46 and 0.65 respectively were used as standard compounds (Fig.4.9). All these compounds were detected on TLC plate on the corresponding Rf values.

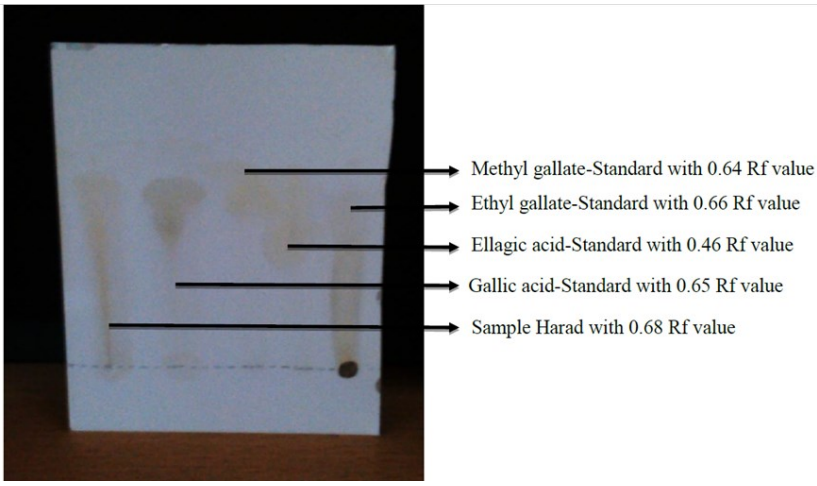


Fig. 4.9: *TLC analysis of methanolic fruit extract of Terminalia chebula*

3.9.2 LC-MS analysis of Methanolic Fruit extract of T. chebula

Further LC-MS analysis of methanolic fruit extract of *T. chebula* was performed to confirm the presence of Gallic acid, Methyl gallate, Ethyl gallate and Ellagic acid in that extract. The peaks obtained in mass spectrum confirmed the presence of Gallic acid (170.12), Methyl gallate (184.12), Ethyl gallate (198.17) and Ellagic acid (302.19) (Fig 4.11).

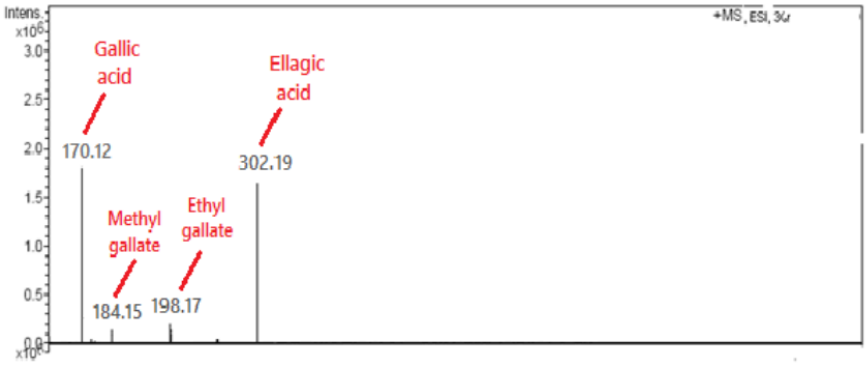


Fig. 4.11: *LCMS analysis of fruit extract of Terminalia chebula showing the peaks of Gallic acid, Methyl gallate, Ethyl gallate and Ellagic acid.*

3.10. Bioassay guided fractionation of fruit extract of *T. chebula*

Bioassay guided fractionation of the methanolic fruit extract of *T. chebula* was performed by using Hexane, Water, Chloroform, Ethyl acetate and Acetonitrile. The compounds were separated by using column chromatography. The synergistic effect was studied for all columns and the column showing synergistic effect was further characterized to identify the bioactive compound responsible for synergistic activity.

3.11. Characterization of the compound isolated from methanolic fruit extract of *T. chebula*

The bioactive compound isolated from *T. chebula* was characterized by its physical properties, LC-MS and NMR studies.

Table 4.14. Physical properties of the compound showing synergistic effect as an EPI for MDR strains of *P.aeruginosa*

S.no.	Physical properties	Results
1.	Name of compound	Ethyl gallate
2.	Color	White
3.	Melting Point	150°C
4.	Solubility	Water soluble

The physical properties of the isolated compound were matching to those of Ethyl gallate.

3.11.1. LC-MS analysis of the isolated compound

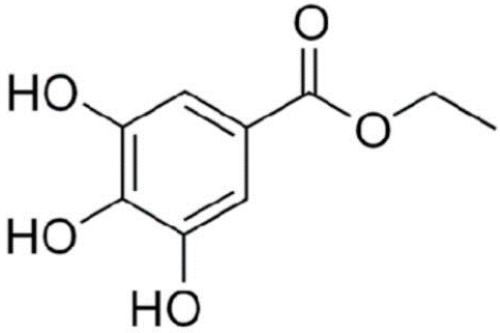
The LC-MS analysis of the isolated compound was performed to confirm the presence of ethyl gallate in methanolic fruit extract of *T. chebula.* The characteristic peak of ethyl gallate was detected at m/z 198.17 which confirmed the isolated compound as ethyl gallate (Fig4.13).

Fig. 4.13: *LCMS analysis of active compound present in Terminalia chebula.*

4.22 NMR, 2D & C13 of active Compound present in *Terminalia chebula*

Active compound present in *Terminalia chebula* was further identified by NMR, 2D and C13 (Fig 4.15, 4.16 and 4.17).

1.1HNMR (400 MHz,δ,CDCl3,TMS=0):1.2700(3H, d, -3H, J=5.88Hz), 4.2169(2H, dd, 2-H, J=9365 Hz), 6.9030(2H, s,2', 6'-H),**8.9365**(3-OH, s, 3', 4'.5'.-H), Exchangeable with D2O



Ethyl 3,4,5-trihydroxybenzoate

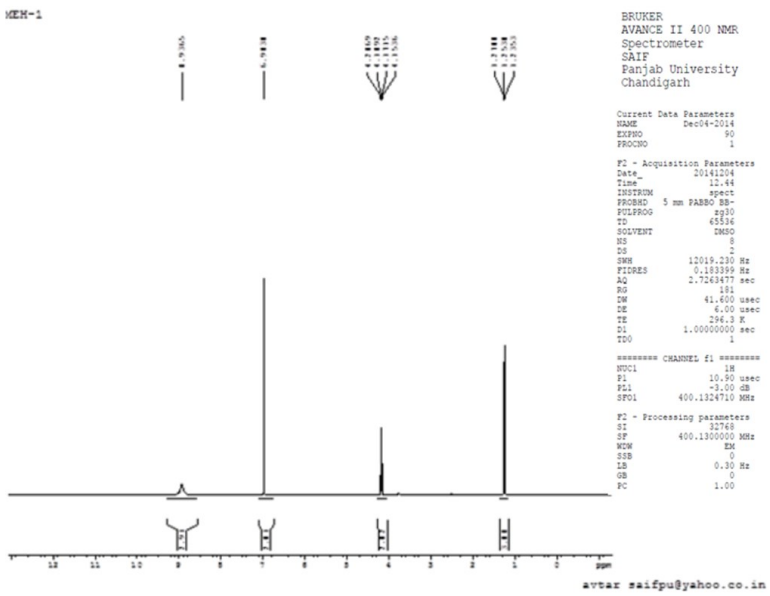


Fig 4.15: *H1NMR of Ethyl gallate*

The structure was established by heteronuclear 2D NMR. Each point simultaneously corresponds to H1 and C13 chemical shift value. (Fig. 4.16)

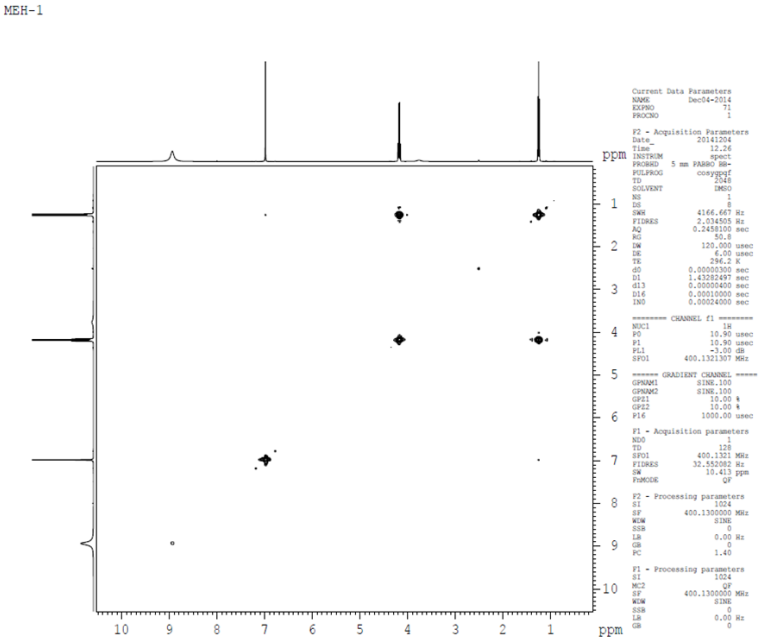


Fig. 4.16: 2D of Ethyl gallate

3.13C-NMR of Ethyl gallate

13C-NMR (400 MHz δ, DMSO, TMS=0): C-1, 165.49, C-3'.5',145.22, C-4',131.99, C-1', 119.32 C-2', 6',109.51, CH2-59.15 5 CH3, 14.96

From above observation of LCMS,1HNMR,13C-NMR, and 2-D NMR ethyl gallate has been identified and structure was established. (Fig. 4.17)

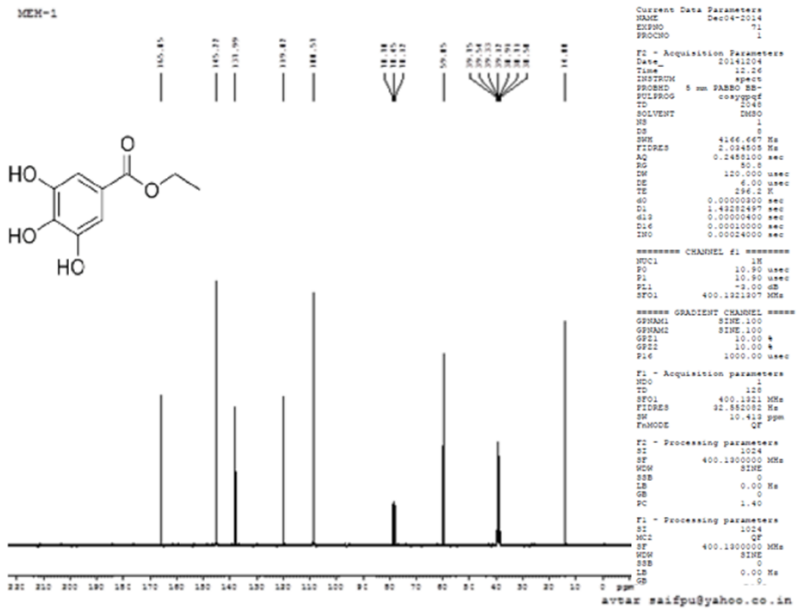


Fig. 4.17: ^{13}C-NMR of Ethyl gallate

4.24: EtBr assay of Ethyl gallate Compound for EPI activity

Active compound isolated from methanolic fruit extract of *Terminalia chebula* was further analysed for its EPI activity by EtBr assay. In this assay the active compound (ethyl gallate), showed EPI activity for *P. aeruginosa* at a concentration of 1000µg/mL. Effect of Ethyl gallate on accumulation of Ethidium bromide at a concentration of 1000ug/ml and 100ug/ml by *P. aeruginosa* overexpressing strain R4 (PT629) (Fig. 4.21)

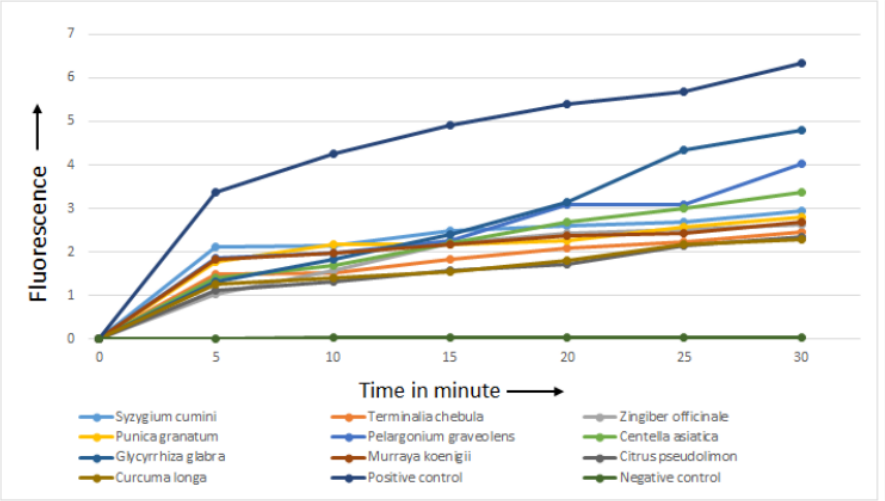


Fig. 4.21: *Effect of Ethyl gallate on accumulation of Ethidium bromide at a concentration of 1000ug/ml by P. aeruginosa overexpressing strain R4 (PT629).*

4.25: Synergistic effect of Plant extracts with Antibiotics

Ethyl gallate and gallic acid has shown potent synergistic effect with the antibiotics. The 1000 µg/mL concentration of ethyl gallate was observed effective with above mentioned antibiotics and effectively decreased the minimum inhibitory concentration of antibiotics (Table 4.16). Further synergism was determined by FIC (Fractional inhibitory concentration) determining method in which first the MIC of all antibiotics and ethyl gallate was determined alone then in combination (Table 4.17). Ethyl gallate has shown synergistic activity with all the three antibiotics (ciprofloxacin, tetracycline and chloramphenicol) for *P. aeruginosa*. Hence, ethyl gallate can be used as an EPI against *P. aeruginosa*.

Table-4.16: MIC of ciprofloxacin, tetracycline and chloramphenicol for *P. aeruginosa* in µg/mL with or wihout Ethyl gallate

S. no.	Name of the strain	MIC of Plant extract (*Terminalia chebula*) (1000 µg/mL)		MIC of Ciprofloxacin		MIC of Plant extract (*Terminalia chebula*) (1000 µg/mL)		MIC of Tetracycline		MIC of Plant extract (*Terminalia chebula*) (1000 µg/mL)		MIC of Chloramphenicol	
		Without Cipro-floxacin	With cipro-floxacin	Without Plant extract	With Plant extract	Witout tetracycline	With tetracycline	Without Plant extract	With Plant extract	Witout Chloram-phenicol	With Chloram-phenicol	Without Plant extract	With Plant extract
1.	R1-Knockout TETR-T (S)	0.06*	0.06*	0.125*	0.125*	0.06*	0.05*	0.125*	0.125*	0.06*	0.03*	2*	2*
2.	R2-Over expressing strain TETR (R)	32	8	32	8	32	8	1	0.125	32	4	2	0.5
3.	R3-Wild type strain PAO1 (R)	0.06	0.003	0.06	0.003	0.06	0.01	2	0.125	0.06	0.01	2	0.5
4.	R4-Over expressing strain PT629 (R)	8	4	2	0.5	8	2	4	0.5	8	0.5	2	1
5.	Standard strain MTCC-471(S)	0.06*	0.06*	0.125*	0.125*	0.06*	0.06*	0.125*	0.125*	0.06*	0.05*	1*	1*
6.	Ps3-Clinical strain (R)	0.5	0.125	0.125	0.06	0.5	0.3	0.5	0.125	0.5	0.125	2	1
7.	PsT10-Clinical strain (R)	2	0.125	0.125	0.06	2	0.5	0.5	0.125	2	0.125	1	0.5
8.	Ps11-Clinical strain(R)	2	1	2	0.5	2	0.5	2	0.5	2	1	2	0.5

Table 4.17: Synergistic activity of Ethyl Gallate compound with ciprofloxacin, tetracycline and chloramphenicol

Name of the strain of *P. aeruginosa*	FIC value with ciprofloxacin	Synergistic activity with ciprofloxacin	FIC value with tetracycline	Synergistic activity with Tetracycline	FIC value with chloramphenicol	Synergistic activity with chloramphenicol
R1-Knockout TETR-T (S)	2	Indifferent	1.83	Indifferent	1.5	Indifferent
R2-Over expressing strain TETR (R)	0.5	Synergistic	0.375	Synergistic	0.37	Synergistic
R3-Wild type strain PAO1 (R)	0.1	Synergistic	0.22	Synergistic	0.41	Synergistic
R4-Over expressing strain PT629 (R)	0.75	Synergistic	0.375	Synergistic	0.56	Synergistic
Standard strain MTCC-471(S)	2	Indifferent	2	Indifferent	1.83	Indifferent
Ps3-Clinical strain (R)	0.73	Synergistic	0.85	Synergistic	0.50	Synergistic
PsT10-Clinical strain (R)	0.375	Synergistic	0.50	Synergistic	0.56	Synergistic
Ps11-Clinical strain(R)	0.73	Synergistic	0.5	Synergistic	0.75	Synergistic

Discussion

Pseudomonas aeruginosa has become increasingly recognized as an emerging opportunistic pathogen of clinical relevance. While the reduced uptake likely does limit the access of antimicrobials to their targets within the cells, it is dependent upon additional resistance mechanisms such as drug efflux (Ma et al., 1994). Addressing antibiotic resistance requires a multifaceted approach to reduce inappropriate use, prevent disease transmission, and to develop novel/ alternative therapy for it. Therefore, the present study was aimed to explore the plants as a source of bioactive molecules which act as an efflux pump Inhibitor for resistant micro pathogens and enhance the sensitivity towards resistant antibiotics.

Ethyl gallate as a putative EPI

Bioactive compound isolated from methanolic fruit extract of *T. chebula* was further analyzed for its EPI activity by EtBr assay. We have observed potent EPI activity of Ethyl gallate against MexAB-oprM efflux pump mediated multidrug resistant strains of *P. aeruginosa* at a concentration of 1000µg/mL. Similarly, Sibanda *et al*., (2007) demonstrated the resistance modifying activity of Ethyl gallate extracted from *P. granatum* fruit peels against methicillin resistant gram-positive bacteria *S. aureus*. The present study is suggested that the Ethyl gallate is a potent efflux pump inhibitor against gram negative bacteria containing MexAB-oprM efflux pump to develop resistance against group A antibiotics as well as the previous studies has also established its EPI activity against gram positive bacteria which developed NorA efflux pump mediated multidrug resistance.

The emergence of multidrug resistance in gram negative bacteria is a major challenge and the control of these pathogenic bacteria is difficult by existing control measures. Plants are the major sources of bioactive molecules which can control the pathogenic organism. Ethyl gallate extracted from plants as a bioactive molecule have potent efflux pump inhibitory activity against multi drug-resistant gram-negative bacteria *P. aeruginosa* which contains MexAB-oprM efflux pump to develop resistance against group A antibiotics. Both plants derived molecules ethyl gallate have synergistic effect in-combination with Group A antibiotic and significantly lower down the MIC and enhance the sensitivity of theses resistant antibiotic up to lethal level suggested that Ethyl gallate may be used as a future molecule as an putative efflux pump inhibitor in combination to resistant antibiotics for the treatment of multi drug resistant strains of *P. aeruginosa.*

The present study produced a preliminary data, and the number of strains were small, the large number of MDR strains is required to make any conclusion.

Summary and Conclusion

The need to combat microbial resistance to antibiotics is an increasing global concern. With the emergence of multidrug resistant organisms, combining the plants and antibiotics against resistance bacteria becomes necessary. Efflux related multidrug resistance (MDR) is a significant means by which bacteria can evade the effects of selected antimicrobial agents. *Pseudomonas aeruginosa*is not an obligate parasite, but the species of *Pseudomonad* commonly associated with human diseases. *P. aeruginosa* is gram-negative microorganism that have been shown to exhibit resistance to a wide range of commonly available antibiotics. The aim of the present study was to identify the Efflux pump inhibitors for multidrug resistance Gram negative bacteria from plant sources and study the synergistic effect of characterized EPI with resistant antibiotics in MDR strains of Gram-negative bacteria *P. aeruginosa*. The aim was achieved by using following objectives. A total of 100 clinical isolates of *P. aeruginosa* were collected from Gian Sagar Medical College and Hospital, Rajpura, Distt. Patiala, Punjab (India). Four control strains of *P. aeruginosa* including, one wild type and three MexAB-oprM efflux pump knockouts/Overexpressing strains were procured from Dr. Thilo Kohler, University of Geneva, and Department of Microbiology and Molecular Medicine Genève, Switzerland. One standard sensitive strain of *P. aeruginosa* MTCC-741 was obtained from IMTECH, Chandigarh. All the clinical isolates of *P. aeruginosa* were characterized by morphologically and biochemically. Further all the 100 clinical isolates were processed for antibiotic susceptibility assay. The antibiotics were divided into two groups based on the substrates and non- substrates of MexAB -oprM efflux pump of *P. aeruginosa*. Group A contains the antibiotics which are refluxed out by MexAB-oprM efflux pump of *P. aeruginosa* and group B contains the antibiotics which are not effluxed out by MexAB-oprM efflux pump of *P. aeruginosa*. The EtBr Agar Cartwheel assay was done for determination of MDR phenotype. This assay confirms the presence or absence of efflux pumps in the bacterial strains. A total of 40 medicinal plants were collected from University of Horticulture and Forestry Nauni, Solan (HP), Arya Vastu Bhandar Dehradoon (Uttrakhand) and Physical Garden of Shoolini University, Solan (HP). The plants collected from Arya vastu bhanda and Shoolini University were authenticated by University of Horticulture and Forestry Nauni, Solan (HP).

All the 40 plants were selected for Efflux pump inhibitory activity. EPI activity was evaluated by Berberine Potentiation assay and Ethidium bromide assay. Berberine works as an efflux pump substrate and inhibits growth of bacteria in the presence of an EPI. There for Berberine is used as a marker to find out the presence of an EPI in plant extracts and Ethidium Bromide is a florescent dye and gets accumulated in MDR efflux pump containing bacteria with an EPI and shows fluorescence after accumulation. Hence, it is also used as a marker for the detection of an EPI in plant extracts. Berberine Potentiation assay and Ethidium bromide assay was performed for methanolic extracts of all 37 plants and for oil of three plants. Out of 40 plants only 10 plant extracts (*T. chebula*, *S. cumini, M. koenigii, Z. officinale, C.pseudolimon, C.asiatica, P.granatum, G.glabra, C. longa, P.graveolens*) enhanced the accumulation of ethidium bromide at 1000 µg/ml concentration and inhibits of efflux pumps activity of the bacterial cells. In addition to this, Berberine control the bacterial growth with all 10 plants extracts. The observation of both assays, Berberine Potentiation assay and Ethidium bromide assay indicates that all these plants have potent EPI activity and a potential source of a bioactive molecule which has EPI activity. Further the synergistic activity for all 10 plants was evaluated in combination with Group-A antibiotics (Ciprofloxacin, Tetracycline and Chloramphenicol). The assay was determined that out of 10 plants only 1 plant extracts, *T. chebula* (Fruits) shown synergistic activity in combination with group A antibiotics (Ciprofloxacin, Tetracycline and Chloramphenicol), and enhanced the sensitivity of these antibiotics used as a substrate for MexAB-oprM efflux pump for *P. aeruginosa*. Furthermore, *T. chebula* plant extracts were used to isolate bioactive compounds responsible for synergistic effect with Group A anitbotics. Isolation and characterization of bioactive compound was done by extracting the powdered dry fruits of *T. chebula* with methanol and followed by chemical fractionation. Then column chromatography was performed on silica gel to extract the compound. Phytochemical analysis and TLC of *T. chebula* fruit extract was done with various solvents depending on their polarity with different Standards.

The fraction was then characterized to know the physical properties of bioactive molecule like colour, solubility, melting point and the molecular weight of bioactive molecule was determined by LCMS then the structure was elucidated by NMR. Further, EPI activity of the bioactive compound extracted from *T. chebula* was performed with two different concentrations (100 µg/ml and 1000 µg/ml) by EtBr assay.

Bioactive compound isolated from methanolic fruit extract of *T. chebula* was further analyzed for its EPI activity by EtBr assay and was also evaluated for synergistic effect with the antibiotics. All 100 clinical isolates of *P. aeruginosa* were collected and maintained in nutrient broth followed by culturing in nutrient agar. All the strains were found Gram negative bacilli with Slimy, opaque, irregular colonies with earthy smell on nutrient agar media and showing positive biochemical test of Catalase, Oxidase, Citrate, Nitrate, Motility and acid production in glucose. All the 100 clinical isolates have shown the characteristics features of *P. aeruginosa*. Drug susceptibility assay was performed for all the 100 isolates of *P. aeruginosa*. Nighty percent clinical isolates were found resistant at least for one antibiotic used for sensitivity assay. Out these 6 isolates were found multi drug resistant against more than two antibiotics. Out of 6 MDR strains 3 strains were found resistant to group A antibiotics even after repeated attempt and sensitive to group B antibiotics. All three clinical MDR isolates and three control strains including one wild type strain (R3-PAO1), two mexAB-oprM efflux pump overexpressing strain of *P. aeruginosa*, (R2-TETR and R4- PT629) has shown very high MIC value *i.e* 128 µg/mL. While the sensitive control strains have shown very low MIC value *i.e* 0.003 µg. The significant decrease was observed in MIC values of antibiotics up to 0.003 µg/mL in combination with control efflux pump inhibitor CCCP in all resistant strains, while the MIC of control sensitive strains (1 MTCC 741 and knockout strain, R1-TETR-T) without efflux pump was not changed and remain constant. The observations of the present study again suggested that the drug resistance in MDR clinical isolates is mediated by an active efflux pump which effluxes out the accumulated drug. Out of 40 plants extracts only 10 plant extracts (*T. chebula*, *S. cumini, M. koenigii, Z. officinale, C. pseudolimon, C. asiatica, P. granatum, G. glabra, C. longa, P. graveolens*) enhanced the accumulation of Ethidium bromide and inhibited its efflux from cells. Furthermore, the observations of Berberine assay, again confirmed the presence of EPI compounds in all ten plants as all extracts have shown EPI activity with Berberine. The observations of both assays Ethidium bromide as well as Berberine assay suggested that these plants have bioactive compounds which has EPI activity and may be proved as promising candidates bearing EPI compounds. All the 10 plants were subjected to evaluate their synergistic activity in combination with group A antibiotics. Out of 10 plants only the methonolic plant extracts of *T. chebula* (Fruits) has shown synergistic activity with three antibiotics (Ciprofloxacin, Tetracycline and Chloramphenicol) belongs to group A, which are exclusively effluxed out by MexAB-oprM efflux pump against *P. aeruginosa*. The methonolic extracts of these two plants enhanced the sensitivity of *Ciproflixacine, tetracycline and Chloremphenicol* up to lethal dose and control the growth of bacteria.

Then the bioactive compound was extracted from *T. chebula* by using TLC, Bioassay guided fractionation and finally by column chromatography. The bioactive compound extracted from T. chebula has molecular weight 198.17 and melting point 150° C with empirical formula C9H10O5. Hence the compound was identified as Ethyl gallate. This phenolic compound has three OH groups.

EPI activity of Ethyl gallate was performed by Ethidium bromide assay at two different concentrations i.e. 100µg/mL and 1000 µg/mL. The potent efflux pump inhibitory activity was observed on 1000 µg/mL. Further, Ethyl gallate was used in combination with group A antibiotic to know the synergistic activity of the compound. Synergism was determined by FIC method. The compound, Ethyl gallate have shown significantly very high synergistic activity with all three (Chloramphenicol, Tetracycline and Ciprofloxacin) group A antibiotic against MDR strains of *P. aeruginosa* and effectively control the growth of bacteria in-vitro and significantly decreased the MIC of antibiotics. The present study suggested that the Ethyl gallate may be used as a putative EPI against Gram negative bacteria and may be tried for clinical use. The emergence of multidrug resistance in Gram negative bacteria is a major challenge and the control of these pathogenic bacteria is difficult by existing control measures. Ethyl gallate may be used as a future molecule as a putative efflux pump inhibitor in combination with antibiotics for the treatment of multi drug resistant strains of *P. aeruginosa*. The present study produced a preliminary data, and the number of strains was small, the large number of MDR strains is required to make any concrete conclusion. But the controls used in this study were standard strains which were genetically confirmed for the activity of efflux pumps in bacteria. Therefore, it may be assumed to a strong possibility of that the Ethyl gallate is a potent efflux pump inhibitor against gram negative bacteria containing MexAB-oprM efflux pump which responsible to develop resistance against group A antibiotics in *P. aeruginosa*. The previously reported EPIs have EPI activity against Gram positive bacteria the dose required for EPI activity is toxic, hence these efflux pump inhibitors may be found helpful to demolish the efflux pumps in Gram negative bacteria and to overcome the development of antibiotic resistance.

Conflict of Interest

The authors declare no conflicts of interest.

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Citation: Kaur N, Ralhan R, Farooq U. Ethyl Gallate: A Potent Inhibitor of MexAB-OprM Efflux Pump in Multidrug-Resistant Pseudomonas aeruginosa. *SVOA Microbiology 2024,* 5:5, 153-170. doi:10.58624 SVOAMB.2024.05.054