



**ESBL genes detected in *Pseudomonas aeruginosa* isolated from dental sources**

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ABSTRACT

Antibiotic resistance is a major crisis during the treatment of nosocomial infections especially in the 20<sup>th</sup> century. Among some common microbes *Pseudomonas aeruginosa* is seen to spontaneously develop into multidrug resistant strain (MDR). *Pseudomonas aeruginosa* has developed to be a multidrug resistant strain to show resistance towards many common antibiotics. Both antibiotics and natural sources individually have not been able to inhibit the virulent growth of the microbe because of which the infection persists. An in-silico approach to find out which phytochemical compounds have could inhibit the MexAB efflux pump, has been studied. As, this system that has been a frequently reported reason for antibiotic resistance in *Pseudomonas aeruginosa*. Ligands present in the plant *Wrightia tinctoria*, have been reported previously for inhibitory activity against *Pseudomonas aeruginosa*. These were used to dock with the efflux pump in study. Quercetin 3-sophoroside and quercetin glycoside has shown best pose with this particular receptor. The binding energy was recorded to be -141.93J. In this report, a resistant strain of *P. aeruginosa* isolated from dental wounds is targeted to find out the possible mechanism of resistance present in it. The research could target two such mechanisms- efflux pump activity and resistive genes.

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## 1. Introduction

For the past 20 years, continuous changes in infection control practises, health care and antimicrobial use during resistance is said to have influenced the frequency of Gram-negative organisms to be associated with nosocomial infections. The most frequently reported types include- Surgical site infection (SSI), Urinary Tract Infections (UTI) and Bloodstream Infections (BSI). Research in 2003 showed that 23.8% of BSI, 33.8% of SSI and 71.1% of UTI is caused by Gram- negative Bacilli. (Robert and Jonathan, 2005). Antimicrobial resistance is the developed ability of microorganisms to resist the activity of antibiotics such as antibacterials, antivirals, and so on. Such a threat has evolved due to unnecessary or over use of common antibiotics against diseases. Antimicrobial resistance hence has led to ineffectiveness of some common antibiotics while treating such infections. As a result, infection persists, spreads and even has a good chance of turning into an outbreak. (WHO newsletter no.32, 2017)

Gram-negative bacilli infections have characteristics that require attention. These microorganisms can easily mutate genes that are responsible for the development of mechanisms to show antibiotic resistance in the presence of a selection pressure. Moreover, they have a number of resistance mechanisms that can be differentiated in a vague manner as- those that show multiple mechanisms against the same antibiotics and the other using a single mechanism to affect many antibiotics (Anton and David, 2010). A range of Gram-negative organisms are responsible for hospital- acquired infections, the Enterobacteriaceae Family being the most commonly identified group overall. However, multidrug organisms, including *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, and ESBL-producing or Carbapenemase-producing *Enterobacteriaceae*, are increasingly being

reported worldwide (Helen et al., 2009). *Pseudomonas aeruginosa* is Gram-negative, rod-shaped, asporogenous, and monoflagellated bacterium. *P. aeruginosa* grows well at 25°C to 37°C. *Pseudomonas aeruginosa* is a ubiquitous microorganism and has the ability to survive under a variety of environmental conditions. It not only causes disease in plants and animals, but also in patients suffering from severe burns and cystic fibrosis (CF) (Fei et al., 2017). Due to its treatment with a wide range of antibiotics today it has developed into a resistant strain showing multidrug resistance against some common antibiotics. Antibiotic resistance in *P. aeruginosa* is shown through many cell survival processes such as biofilm formation, efflux pump activity, or in many cases due to resistance phenotype expressing genes. The efflux pumps possessed by *Pseudomonas aeruginosa* are *AmR*, which is expressed due to the *AmR AB* genes.

The RND systems of this microbe also effectively execute resistance through efflux pumps such as *MeX AB-OprM*, *Mex XY-OprM*, *MexCD-OprJ*, *MexEF-OprN*, *MexJK*, *MexGHI-OpmD*, *MexVW*, *MexPQ-OpmE*, *MexMN*, and *TriABC* (Jürg Dreier, Paolo Ruggerone, et al.,2015) One of which *Mex AB-OprM* is the most important as it provides intrinsic resistance to a broad spectrum of antibiotics. The genes involved in the synthesis of the Mex AB protein get frequently expressed in the presence of common antibiotics especially  $\beta$ -lactams. (Poole et al., 1993). Research from late 90's on phytochemistry has brought to light the synergetic activity of natural compounds present in plants with common antibiotics against AMR. (Michael et al., 2007) Further interest in this area has brought the idea of doing in silico research on the phytochemicals present in Indian Medicinal Plants that showed such synergetic activity with the common antibiotics against multidrug resistant strains of *Pseudomonas aeruginosa* (MDRPA). Furthermore,

*Pseudomonas aeruginosa* is one of the most important causes of Gram-negative infection, especially in immunocompromised individuals and patients undergoing treatment in hospitals. It is the most common pathogen isolated from patients who have been hospitalized for more than 1 week (Marcus et al., 20018). MDRPA has been previously observed to show resistance to a number of antibiotics including Ciprofloxacin, Ceftazidime, Imipenem and Piperacillin during a retrospective study conducted at Beth Israel Deaconess Medical Centre. Some isolates even showed resistance to aminoglycosides such as Gentamicin and Tobramycin. The same study also illustrated that the most frequent sites of Infection were-soft tissue, bone, lungs, UT, all characterised by prolonged infections, multiple and lengthy antipseudomonal antibiotic exposure and long hospitalisation (Laura et al., 20114).

This paper deals with such a hospital isolated strain of *Pseudomonas aeruginosa* that showed delayed cure, and poor treatment with many antibiotics, isolated at Balaji Dental College and Hospitals, Chennai. The Susceptibility was once again tested with some common antibiotics and the reason for resistance was determined targeting the various mechanisms of resistance.

The pervious researchers have reported an expanded program on immunization (EPI) activity against *Pseudomonas aeruginosa* resistant strains. Based on the availability and EPI activity of *Wrightia tinctoria* was selected as the working sample for this research. *Wrightia tinctoria* also known as Sweet Indrajao, Dyer's Oleander, Vetpaalai, belongs to the Apocynaceae family. It is available all over the world and abundantly in parts of South-east Asia mostly Vietnam and India. This tree in South India is used as a Jaundice curative. Apart from this, the plant has been recognized to have a lot of anti-inflammatory properties. Studies have suggested

that different parts of the plant are said to possess different phytochemical compounds that play a significant role in the treatment of various bacterial and fungal infections (Gangola et al., 2017; Ekins et al., 2007).

Types of resistance mechanisms that can be shown by a microorganism especially the Gram-negative strain has been illustrated shows in Fig 1.

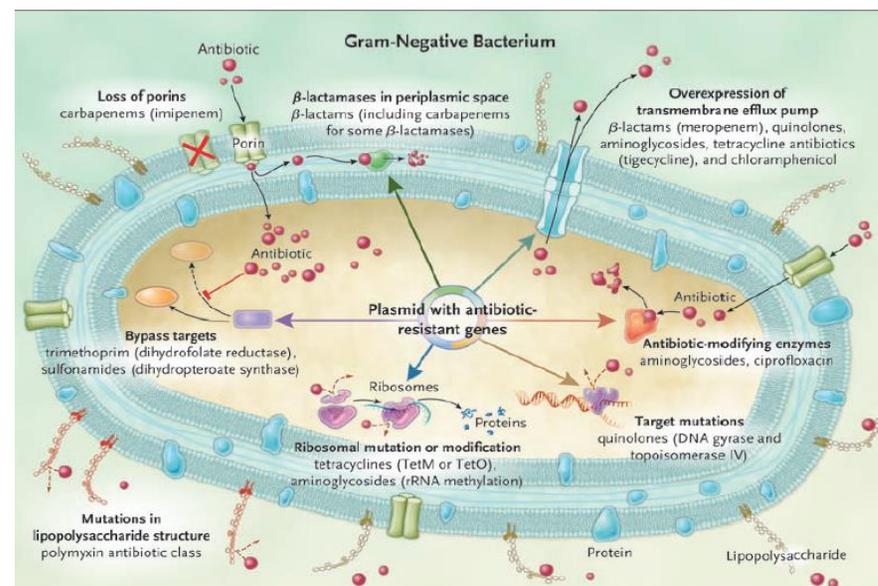


Fig 1. Various mechanisms of resistance developed by Gram-negative bacterium such as Overexpression of transmembrane efflux pump, mutations in Lipopolysaccharide structure, Loss of porins,  $\beta$ -lactamase production (Anton et al., 2010).

The objective of this research is to study whether the phytochemicals present in *Wrightia tinctoria* has the efflux pump inhibitory activity against the multidrug resistant strain of

*Pseudomonas aeruginosa* and find out which compound could be responsible for the inhibitory activity of the *MexAB-OprM* efflux pump shown through in-silico studies.

## 2. Materials and methods

### 2.1. Virtual visualization

Visualization of the phytochemicals and the proteins that constitute the *MexAB* pump, in a 3dimensional view, was possible with the help of Biovia Discovery Studio. It's software that helps create and display the chemical and biomolecules virtually. With Biovia one can research and conduct test to hypothesize prior to conducting wet lab experiments which consumes effort, time and fund. (<https://www.3dsbiovia.com/products/collaborative-science/biovia-discovery-studio/>).

### 2.2. Ligand identification and Preparation for Screening

A total of 47 phytochemical compounds reported to be present in the plant sample *Wrightia tinctoria* were selected from literature study. The 3dimensional structures of these phytochemical compounds were retrieved from the database called PubChem. PubChem is a database of chemical compounds that helps us to know achieve the activities of those compounds in biological assays. This database is maintained by the National Centre of Biotechnology Information (NCBI). Structures, IUPAC names, and much other information regarding the chemical compounds under study can be obtained from this database. (<https://pubchem.ncbi.nlm.nih.gov/>)

The 3dimensional structures of these compounds were achieved from this database and were downloaded in SDF format.

### 2.3. Receptor structure retrieval

The protein involved in the efflux pump activity in the *Pseudomonas aeruginosa* was reported to be *MexAB-OprM* which is expressed due to the *OprM* genes present in the bacteria (Michael et al., 2007). The structure of this protein complex was achieved from the software named Protein Data Bank. The structure (Pdb id: 1LNW) was downloaded and converted to .mol2 format to be used for molecular docking as our receptor with which all the selected phytochemical compounds present in *Wrightia tinctoria* would bind as ligands.

### 2.4. Protein data bank

This is a database that contains three-dimensional structures and other data of complex and large molecules especially proteins and nucleic acids. This database acts as a resource in these areas of biology where structure plays a vital role in the activity under study. (<https://www.rcsb.org/>)

### 2.5. Molecular Docking

The *MexAB-OprM* protein structure along with the ligands, all already converted to .mol2 format were input into docking software named iGEMDOCK.

### 2.6. iGEMDOCK

iGEMDOCK performs structure based virtual screening and post screening analysis of input which is usually a combination of receptors and ligands and also performs emergent tasks in computer aided drug designing. It acts as an automatic and graphical drug discovery system which does screening, post-analysis and visualization of virtually performed screening where prediction of how chemical molecules bind to a receptor of known three-dimensional structure is executed.

(<http://gemdock.life.nctu.edu.tw/dock/download/iGEMDOCK-guide.pdf>). The docking process was run with 47 ligands and 1 receptor to find out the best poses in post-analysis.

### 2.6.1. Sample collection

Seeds of *Wrightia tinctoria* were collected from the forest research department, Vandalur, Chennai. The seeds were shade dried and stored in cool area.

### 2.6.2. Extract preparation

Seeds of *Wrightia tinctoria* were crushed with mortar and pestle into a coarse powder and used for extraction with the help of solvent treatment. The following solvents of hexane, ethyl acetate and ethanol were used for extraction. 96.2 g by dry weight of the crushed seeds was taken along with 300 mL of hexane. The vessel (500mL conical flask) was covered with aluminium foil and incubated at 37°C for 24 hrs. After that incubation filtered by using of Whatman filte No.3. The extraction was noted and stored under the freezer condition. Sediment was air dried and used for the next solvent extraction with ethyl acetate and then ethanol. The 3 extracts of *W. tinctoria* in hexane, ethyl acetate and ethanol were concentrated by air drying and collected in screw cap vials. The vials were stored at room temperature.

### 2.6.3. Qualitative phytochemistry

All the three (Hexane, Ethyl acetate, and Ethanol) extracts were diluted to a concentration of 1mg/mL and used for phytochemical screening was done to check for the presence of carbohydrates, tannins, saponins, flavonoids, alkaloids, glycosides, cardiac glycosides, terpenoids, phenol, coumarins, steroids/phyto steroids, phlobatanins, and anthraquinones.

### 2.6.4. Antibacterial activity

The antibacterial activity of the extracts was checked individually as follows using agar well diffusion method using extract (Ext) and antibiotic-Tetracycline (Tet) at the concentration of 1mg/mL.

S. No	Sample	Con A	Con B	Con C	Con D	Con E
1.	Plate1	10µl	20µl	10µl	40µl	10µl
		Tet	Ext	Tet+20µl Ext	Ext	Tet+40µl Ext
2.	Plate2	10µl	60µl	10µl	80µl	10µl
		Tet	Ext	Tet+60µl Ext	Ext	Tet+80µl Ext
3.	Plate3	10µl	100µl	10µl	120µl	10µl
		Tet	Ext	Tet+100µl Ext	Ext	Tet+120µl Ext

The same protocol was followed with the other two extracts as Set2 and 3 respectively.

The three well swabbed LB agar plates with test microorganism were used to puncture 5 wells using a standard metal punch of 0.5 cm diameter, for each extract. The wells were labelled as A, B, C, D and E. the five wells were filled in this manner shows in Table1.

### 2.6.5. Efflux pump inhibition analysis

The efflux pump inhibition activity of the extracts was tested using Accumulation Assay. 3 test tubes containing 6mL of fresh sterile LB broth was taken. To this, 100µL of *Pseudomonas aeruginosa* strain broth culture in each tube. 5µl of Tetracycline (1mg/mL) was added to each tube and incubated at 37°C for 24 hrs.

3mL of culture from each test tube was transferred into 3 micro centrifuge tubes labelled as T1, T2, T3.

T1 was centrifuged at 8000 rpm for 5 minutes at  $-4^{\circ}\text{C}$  to obtain the cell free supernatant. T2 was centrifuged at the same rpm and the pellet was subjected to cell lysis by treating with  $100\mu\text{L}$  of 10% SDS. T3 was also subjected to cell lysis in the same manner as T2. The lysed solution was re-centrifuged and the supernatant was collected. Cell lysed samples were made up to 3mL in the micro centrifuge tubes using Phosphate-buffered saline (PBS). The Tetracycline concentration in each sample was determined spectrophotometrically by reading at (380-450) nm. Reading was taken using LB media with  $5\mu\text{L}$  Tetracycline ( $1\text{mg/mL}$ ) as blank and PBS buffer as blank for the cell lysed samples.

#### 2.6.6. Genomic DNA isolation

Total cell DNA of *Pseudomonas aeruginosa* was isolated using 1.5mL of the bacterial culture in LB broth. Phenol: Chloroform method of DNA isolation was used to isolate the DNA with the help of differential solubility technique and the isolated DNA was stored in  $20\mu\text{L}$  TE buffer at  $-20^{\circ}\text{C}$ .

#### 2.6.7. Molecular characterisation

The presence of resistive genes in the isolated DNA of the DRPA was identified by performing PCR with the sample DNA using SHV primers as the template. Presence of the genes was checked by gel documentation through agarose gel electrophoresis.

### 3. Results

#### 3.1. Molecular docking

The phytochemicals that were used for docking are given in the table 2.

#### MexAB-OprM pump:

PDB ID: 1LNW

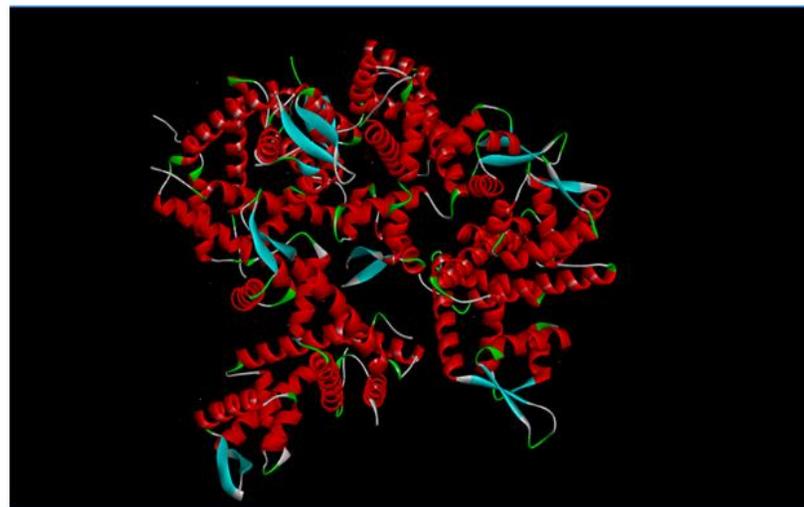


Fig 2 MexAB OprM efflux pump produced by *P. aeruginosa* as a mechanism to develop antibiotic resistance.

It was found that the phytochemical Quercetin 3-sophoroside showed in Fig. 1. the best pose with a total energy of  $-141.931\text{Joules}$  and H-bond value of  $-45.22\text{ Joules}$  on docking with our receptor under study, *MexAB-OprM* efflux pump. However, the top 5 compound that showed good posing with our receptor should also be taken into consideration. The five compounds of Quercetin 3-sophoroside (T.E=  $-141.931\text{J}$  ; H-bond=  $-41.2182\text{J}$ ), Rutin (T.E=  $-128.26\text{J}$  ; H-bond=  $-27.84\text{J}$ ), 9,12,15- Octadecatrienoic acid (T.E=  $-120.07\text{J}$  ; H-bond=  $-17.57\text{J}$ ), Quercetin (T.E=  $-104.62\text{J}$  ; H-bond=  $-30.56\text{J}$ ), Clerosterol (T.E=  $-104.44\text{J}$  ; H-bond=  $-10.43\text{J}$ ).

Table 2.

S.No	Compound name	Source	Molecular formula	PubChem ID	IUPAC name
1.	Quercetin 3-sophoroside	Leaf	<u>C<sub>27</sub>H<sub>30</sub>O<sub>17</sub></u>	44259144	3-[(2S,5S)-4,5-dihydroxy-6-(hydroxymethyl)-3-[(2S,3R,5S)-3,4,5-trihydroxy-6-(hydroxymethyl) oxan-2-yl] oxyoxan-2-yl] oxy-2-(3,4-dihydroxyphenyl)-5,7-dihydroxychromen-4-one
2.	Kaempferol	Leaf	<u>C<sub>15</sub>H<sub>10</sub>O<sub>6</sub></u>	5280863	3,5,7-trihydroxy-2-(4-hydroxyphenyl) chromen-4-one
3.	Quercetin	Leaf	<u>C<sub>15</sub>H<sub>10</sub>O<sub>7</sub></u>	5280343	2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxychromen-4-one
4.	Indirubin	Leaf	<u>C<sub>16</sub>H<sub>10</sub>N<sub>2</sub>O<sub>2</sub></u>	5359405	(3E)-3-(3-oxo-1H-indol-2-ylidene)-1H-indol-2-one
5.	Indole	Leaf	<u>C<sub>8</sub>H<sub>7</sub>N</u>	798	1H-indole
6.	Anthocyanin	Leaf	C <sub>15</sub> H <sub>11</sub> O <sup>+</sup>	145858	2-phenylchromenylium
7.	Calcones	Leaf	<u>C<sub>21</sub>H<sub>18</sub>N<sub>2</sub>O<sub>3</sub>S</u>	6445661	4-amino-N-[4-[(E)-3-oxo-3-phenylprop-1-enyl] phenyl] benzene sulfonamide
8.	Catechin	Leaf	<u>C<sub>15</sub>H<sub>14</sub>O<sub>6</sub></u>	9064	(2R,3S)-2-(3,4-dihydroxyphenyl)-3,4-dihydro-2H-chromene-3,5,7-triol
9.	Isatin	Leaf		7054	1H-indole-2,3-dione

			<u>C<sub>8</sub>H<sub>5</sub>NO<sub>2</sub></u>		
10.	Indigotin	Leaf	<u>C<sub>16</sub>H<sub>10</sub>N<sub>2</sub>O<sub>2</sub></u>	5318432	(2E)-2-(3-oxo-1H-indol-2-ylidene)-1H-indol-3-one
11.	Rutin	Leaf	<u>C<sub>27</sub>H<sub>30</sub>O<sub>16</sub></u>	5280805	2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-3-[(2S,3R,4S,5S,6R)-3,4,5-trihydroxy-6-[[[(2R,3R,4R,5R,6S)-3,4,5-trihydroxy-6-methyloxan-2-yl] oxymethyl] oxan-2-yl] oxochromen-4-one
12.	Desmosterol	Seed	<u>C<sub>27</sub>H<sub>44</sub>O</u>	439577	(3S,8S,9S,10R,13R,14S,17R)-10,13-dimethyl-17-[(2R)-6-methylhept-5-en-2-yl]-2,3,4,7,8,9,11,12,14,15,16,17-dodecahydro-1H-cyclopenta[a]phenanthren-3-ol
13.	Clerosterol	Seed	<u>C<sub>29</sub>H<sub>48</sub>O</u>	5283638	(3S,8S,9S,10R,13R,14S,17R)-17-[(2R,5S)-5-ethyl-6-methylhept-6-en-2-yl]-10,13-dimethyl-2,3,4,7,8,9,11,12,14,15,16,17-dodecahydro-1H-cyclopenta[a]phenanthren-3-ol
14.	9-octadecanoic acid	Seed oil	<u>C<sub>19</sub>H<sub>36</sub>O<sub>2</sub></u>	21677595	8-[(1S,2R)-2-octylcyclopropyl] octanoic acid
15.	Alpha-amyrin	Seed oil	<u>C<sub>30</sub>H<sub>50</sub>O</u>	73170	(3S,4aR,6aR,6bS,8aR,11R,12S,12aR,14aR,14bR)-4,4,6a,6b,8a,11,12,14b-octamethyl-2,3,4a,5,6,7,8,9,10,11,12,12a,14,14a-tetradecahydro-1H-picen-3-ol

16.	Squalene	Seed oil	<u>C<sub>30</sub>H<sub>50</sub></u>	638072	(6E,10E,14E,18E)-2,6,10,15,19,23-hexamethyltetracosane 2,6,10,14,18,22-hexaene
17.	Gamma-tocopherol	Seed oil	<u>C<sub>28</sub>H<sub>48</sub>O<sub>2</sub></u>	92729	(2R)-2,7,8-trimethyl-2-[(4R,8R)-4,8,12-trimethyltridecyl]-3,4-dihydrochromen-6-ol
18.	Campesterol	Seed oil	<u>C<sub>28</sub>H<sub>48</sub>O</u>	173183	(3S,8S,9S,10R,13R,14S,17R)-17-[(2R,5R)-5,6-dimethylheptan-2-yl]-10,13-dimethyl-2,3,4,7,8,9,11,12,14,15,16,17-dodecahydro-1H-cyclopenta[a]phenanthren-3-ol
19.	Betulin	Seed oil	<u>C<sub>30</sub>H<sub>50</sub>O<sub>2</sub></u>	72326	(1R,3aS,5aR,5bR,7aR,9S,11aR,11bR,13aR,13bR)-3a-(hydroxymethyl)-5a,5b,8,8,11a-pentamethyl-1-prop-1-en-2-yl-1,2,3,4,5,6,7,7a,9,10,11,11b,12,13,13a,13b-hexadecahydrocyclopenta[a]chrysen-9-ol
20.	Lupenone	Seed oil	<u>C<sub>30</sub>H<sub>48</sub>O</u>	92158	(1R,3aR,5aR,5bR,7aR,11aR,11bR,13aR,13bR)-3a,5a,5b,8,8,11a-hexamethyl-1-prop-1-en-2-yl-2,3,4,5,6,7,7a,10,11,11b,12,13,13a,13b-tetradecahydro-1H-cyclopenta[a]chrysen-9-one
21.	Phytol	Seed oil	<u>C<sub>20</sub>H<sub>40</sub>O</u>	5280435	(E,7R,11R)-3,7,11,15-tetramethylhexadec-2-en-1-ol
22.	Pentadecanoic acid, ethyl ester	Flower	<u>C<sub>17</sub>H<sub>34</sub>O<sub>2</sub></u>	38762	ethyl pentadecanoate

23.	Dimethyl phthalate	Flower	<u>C<sub>10</sub>H<sub>10</sub>O<sub>4</sub></u>	8554	dimethyl benzene-1,2-dicarboxylate
24.	Hydroquinone	Leaf EO	<u>C<sub>6</sub>H<sub>6</sub>O<sub>2</sub></u>	785	benzene-1,4-diol
25.	9,12,15-octadecatrienoic acid	Leaf EO	<u>C<sub>28</sub>H<sub>34</sub>O<sub>4</sub></u>	57397401	(5,8-dioxonaphthalen-1-yl) (9Z,12Z,15Z)-octadeca-9,12,15-trienoate
26.	2-heptenal	Seed oil	<u>C<sub>7</sub>H<sub>12</sub>O</u>	5283316	(E)-hept-2-enal
27.	Phenol	Leaf EO	<u>C<sub>6</sub>H<sub>6</sub>O</u>	996	Phenol
28.	Benzyl alcohol	Leaf EO	<u>C<sub>7</sub>H<sub>8</sub>O</u>	244	phenyl methanol
29.	n-methyl-n-2-pyridinyl formamide	Leaf EO	<u>C<sub>7</sub>H<sub>8</sub>N<sub>2</sub>O</u>	572611	N-methyl-N-pyridin-2-ylformamide
30.	2-methoxy phenol	Leaf EO	<u>C<sub>7</sub>H<sub>8</sub>O<sub>2</sub></u>		2-methoxyphenol

				460	
31.	2,4-dimethyl phenol	Leaf EO	<u>C<sub>8</sub>H<sub>10</sub>O</u>	7771	2,4-dimethylphenol
32.	4-ethyl phenol	Leaf EO	<u>C<sub>8</sub>H<sub>10</sub>O</u>	31242	4-ethylphenol
33.	3,5-dichloro phenol	Leaf EO	<u>C<sub>6</sub>H<sub>4</sub>Cl<sub>2</sub>O</u>	11571	3,5-dichlorophenol
34.	2,3-dimethyl phenol	Leaf EO	<u>C<sub>8</sub>H<sub>10</sub>O</u>	10687	2,3-dimethylphenol
35.	n-hexadecenoic acid	Leaf EO	<u>C<sub>16</sub>H<sub>32</sub>O<sub>2</sub></u>	985	hexadecenoic acid
36.	2-methoxy 4-methylphenol	Leaf EO	<u>C<sub>8</sub>H<sub>10</sub>O<sub>2</sub></u>	7144	2-methoxy-4-methylphenol
37.	Alpha-cubebene	Leaf EO	<u>C<sub>15</sub>H<sub>24</sub></u>	86609	
38.	Alpha-caryophyllene	Leaf EO	<u>C<sub>15</sub>H<sub>24</sub></u>	5281520	(1E,4E,8E)-2,6,6,9-tetramethylcycloundeca-1,4,8-triene

39.	Butylated hydroxyl toluene	Leaf EO	<u>C<sub>15</sub>H<sub>24</sub>O</u>	31404	2,6-ditert-butyl-4-methylphenol
40.	Dodecanoic acid	Leaf EO	<u>C<sub>12</sub>H<sub>24</sub>O<sub>2</sub></u>	3893	dodecanoic acid
41.	Caryophyllene oxide	Leaf EO	<u>C<sub>15</sub>H<sub>24</sub>O</u>	1742210	
42.	1-ethyl heptylbenzene	Leaf EO	<u>C<sub>13</sub>H<sub>20</sub></u>	14115	heptyl benzene
43.	1-pentyl heptylbenzene	Leaf EO	<u>C<sub>18</sub>H<sub>30</sub></u>	17629	dodecan-6-ylbenzene
44.	3-ethyldecylbenzene	Leaf EO	<u>C<sub>18</sub>H<sub>30</sub></u>	56994984	3-ethyldecylbenzene
45.	Coumarin	Seed	<u>C<sub>9</sub>H<sub>6</sub>O<sub>2</sub></u>	323	chromen-2-one
46.	Anthraquinone	Seed	<u>C<sub>14</sub>H<sub>8</sub>O<sub>2</sub></u>	6780	anthracene-9,10-dione
47.	4-methylphenol	Leaf EO	<u>C<sub>7</sub>H<sub>8</sub>O</u>	2879	4-methylphenol

#Ligand	TotalEnergy	VDW	HBond	Elec	AverConPair
VIGNESH receptor-Quercetin 3-sophoroside-0.pdb	-141.931	-96.7133	-45.2182	0	21.9318
VIGNESH receptor-Rutin-1.pdb	-128.26	-100.422	-27.8376	0	18.186
VIGNESH receptor-9,12,15-Octadecatrienoic acid-0.pdb	-120.073	-102.498	-17.5748	0	22.3125
VIGNESH receptor-Quercetin-1.pdb	-104.621	-74.0587	-30.562	0	23.1818
VIGNESH receptor-Clerosterol-0.pdb	-104.442	-94.0079	-10.4337	0	24.0667
VIGNESH receptor-gamma-Tocopherol-0.pdb	-104.285	-99.2496	-5.03575	0	22.3667
VIGNESH receptor-Indigotin-1.pdb	-103.778	-80.1363	-23.642	0	31.7
VIGNESH receptor-Campesterol-0.pdb	-102.689	-96.3935	-6.29541	0	23.4483
VIGNESH receptor-Indirubin-0.pdb	-101.125	-82.4891	-18.6363	0	30.35
VIGNESH receptor-calcione-1.pdb	-101.116	-87.1161	-14	0	21.8889
VIGNESH receptor-Desmosterol-0.pdb	-100.774	-94.6149	-6.15925	0	24.0357
VIGNESH receptor-Kaempferol-1.pdb	-99.4154	-72.733	-26.6824	0	27.4762
VIGNESH receptor-CATECHIN-1.pdb	-98.176	-69.8837	-28.2923	0	25.9048
VIGNESH receptor-Squalene-1.pdb	-97.0086	-97.0086	0	0	21.1333
VIGNESH receptor-Betulin-1.pdb	-92.9371	-81.3987	-11.5385	0	17.625
VIGNESH receptor-Lupenone-0.pdb	-91.5939	-89.0939	-2.5	0	19.6774
VIGNESH receptor-alpha-Amyrin-0.pdb	-90.9778	-85.2668	-5.71105	0	19.129
VIGNESH receptor-Anthraquinone-1.pdb	-85.3969	-78.4131	-6.98385	0	31.4375
VIGNESH receptor-dodecanoic acid-1.pdb	-85.157	-68.4584	-14	-2.69857	30.5
VIGNESH receptor-Pentadecanoic acid, ethyl ester-0.pdb	-84.5866	-78.0771	-6.50943	0	28.2105
VIGNESH receptor-Phytol-0.pdb	-83.8063	-74.3634	-9.44298	0	22.9524
VIGNESH receptor-Dimethyl Phthalate-1.pdb	-83.5593	-53.5168	-30.0424	0	27.3571
VIGNESH receptor-N-Hexadecanoic acid-0.pdb	-81.6789	-71.182	-10.497	0	26.6667

Fig 3. Table showing the best fit ligand found in *W. tinctoria* against the MexAB OprM pump. The ligands are arranged in ascending order of lowest binding energy

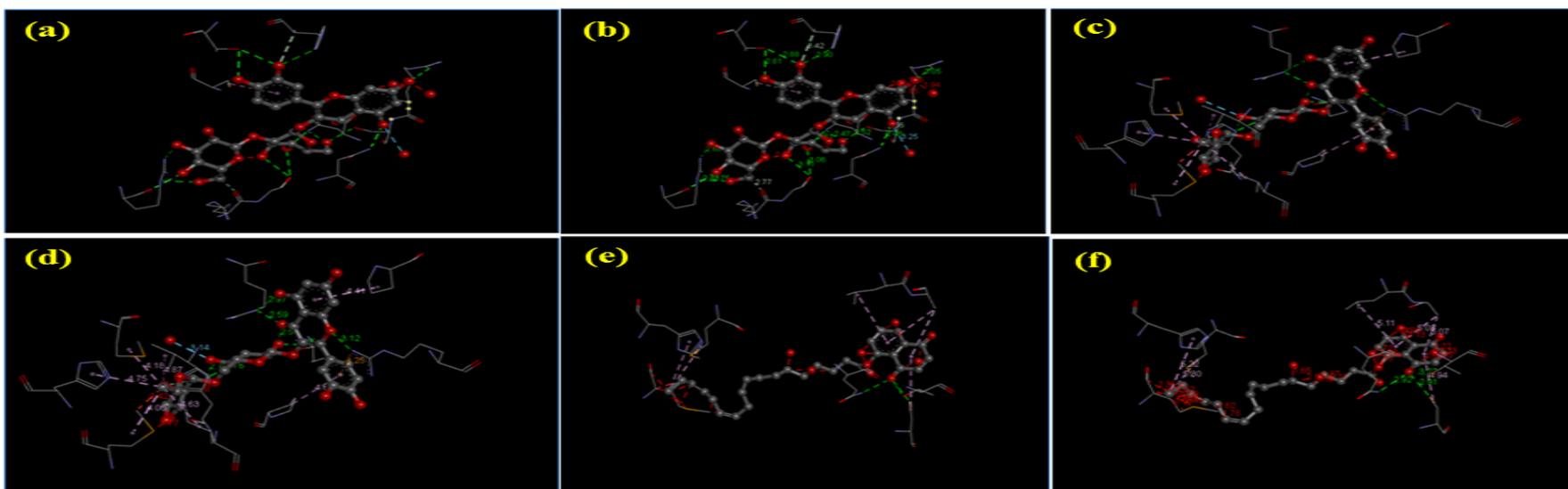


Fig 4. Post docking analysis of Receptor with Quercetin 3-sophoroside, position 1 (a), Post docking analysis of Receptor with Quercetin 3-sophoroside, Position 2 (b), Post docking analysis of Receptor with Rutin, Position 1 (c), Post docking analysis of Receptor with Rutin, Position 2 (d), Post docking analysis of Receptor with 9,12,15-octadecatrienoic acid, Position 1 (e) and Post docking analysis of Receptor with 9,12,15-octadecatrienoic acid. Position 2 (f).

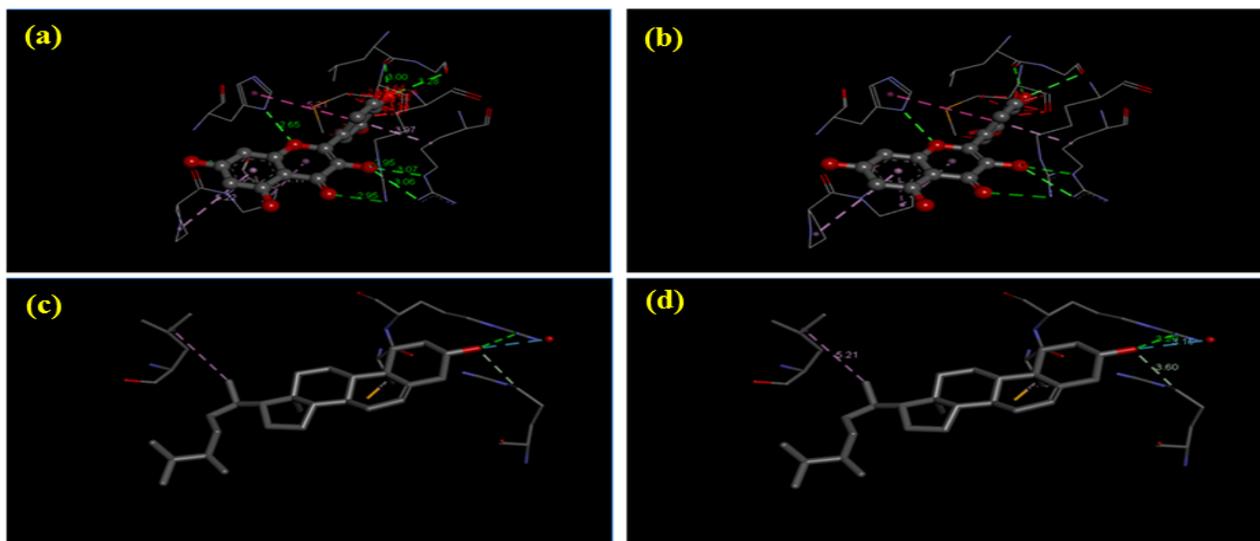


Fig 5. Post docking analysis of Receptor with Quercetin. Position 1, (a), Post docking analysis of Receptor with Quercetin. Position 2 (b), Post docking analysis of Receptor with Clerosterol. Position 1 (c) and Post docking analysis of Receptor with Clerosterol. Position 2 (d).

Out of the top five best poses Quercetin 3-sophoroside showed the best fit with a bond energy of -141.931Joules.

### 3.2. Microbiological & phytochemical assays

The *Wrightia tinctoria* seeds that resembled paddy grains in colour produced a light scarlet red solution on extraction with different solvents. The extracts were observed to turn darker in colour as the extracts concentrated on air drying. The antibiogram showed Fig4 a, that the *P. aeruginosa* isolated was resistant to methicillin and rifampicin but not to ciprofloxacin and streptomycin.

### 3.3. Antibiotic susceptibility test

The well diffusion assay done to check for antibacterial activity showed zones of inhibition around wells where tetracycline was used alone and around wells where it was along with the extract.

Zones were observed in wells which had 10 $\mu$ l Tet, (20 $\mu$ l Ext + 10 $\mu$ lTet), (40 $\mu$ l Ext + 10 $\mu$ l Tet), (60 $\mu$ L Ext +10 $\mu$ l Tet), (80 $\mu$ l Ext +10 $\mu$ l Tet), (100 $\mu$ l Ext + 10 $\mu$ l Tet), (120 $\mu$ l Ext + 10 $\mu$ l Tet).

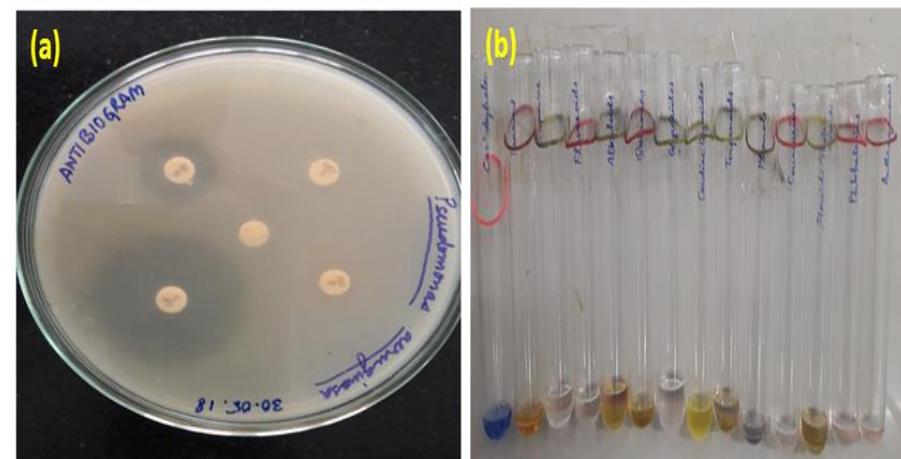


Fig. 6 Antibiogram of *Wrightia tinctoria* seeds extract (a), and (b) phytochemical assays.

These observations were recorded while working with the hexane extract of *W.tinctoria*. The zones observed around well which had both the antibiotic and the extract however, showed larger diameter than with only the antibiotic (Tet). The diameter difference observed was (0.5-1 cm). This may be considered as positive synergistic activity of the plant extracts.

### 3.4. Accumulation assay

Table 4. The spectrophotometric observation of samples from the accumulation assay furnished the results listed in the table 4.

Nanometres	Sample 1	Sample2	Sample3
380	0.0124	-0.1487	-0.0069
390	0.0117	-0.1496	-0.0055
400	0.0124	-0.1527	-0.0062
410	0.0132	-0.1527	-0.0062
420	0.0139	-0.1520	-0.0062
430	0.0145	-0.1527	-0.0056
440	0.0139	-0.1520	-0.0060
450	0.0143	-0.1525	-0.0060

The spectrophotometer readings of absorbance from (380-450) nm show no accumulation of drug in samples 2 and 3. Thus, no drug accumulation indicated absence of an active efflux pump mechanism.

### 3.5. Polymerase chain reaction

The isolated DNA of the resistant *Pseudomonas aeruginosa* was taken for PCR to check for the presence of resistive genes. PCR was done with SHV primer. The PCR product when run through Agarose showed in Fig. 7 amplification. Presence of SHV sequences that belong to the ESBL family was confirmed which also confirms the presence of resistive genes that were capable of producing antibiotic destroying enzymes.

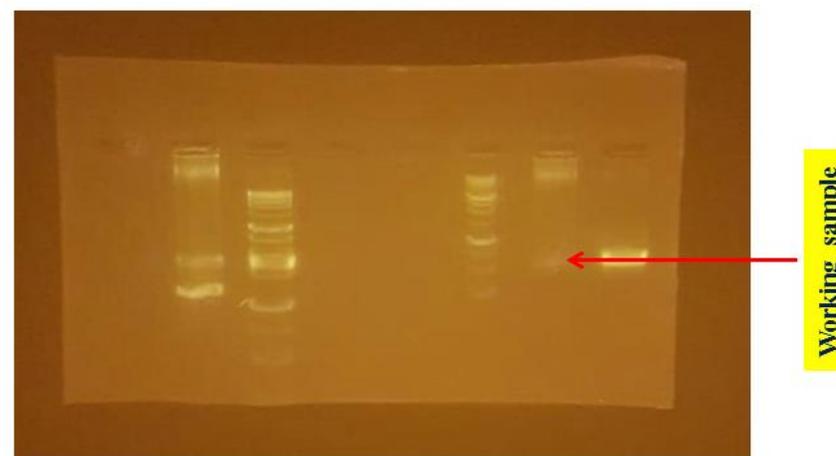


Fig. 7 Agarose gel electrophoresis done post PCR showing the amplification of the SHV gene

### 4. Discussion

In silico analysis was done to find out what possible compounds extracted from natural sources could be used to block the MexAB efflux pump activity shown by the MDR strain of *Pseudomonas aeruginosa*. In silico methods furnished a speedy reporting of such compounds that showed best posing using the method of Molecular Docking. Wet lab assays would have instead taken much more time to complete and conclude with precise results. The receptor that was

chosen for study, the *MexAB* efflux pump, was reported to show multidrug resistance against  $\beta$ -lactams, fluoroquinolones, novobicin, chloramphenicol, macrolides, sulfonamides, trimethoprim, tetracycline and some other detergents and dyes (David et al., 2002). The ligands that showed best interaction with the receptor under study are quercetin 3-sophoroside, rutin, 9,12,15-octadecatrienoic acid, quercetin, clerosterol, and gamma-tocopherol, in the order of increasing binding energy. All these five compounds have been reported to have medicinally important value. Abdollahzadeh et al., 2011, reported that quercetin 3-sophoroside isolated from Korean *Papaver rhoeas* bee pollen showed neurominidase inhibitory activities. A combination of quercetin and rutin has been reported to enhance antibacterial activity of flavonoids, against *Bacillus cereus* and *Salmonella enteritidis* (Hidetoshi et al., 2002). In general, quercetin glycosides isolated from red fruits showed antimicrobial, antiviral activity. (Khatereh and Reza, 2013). A report on phytochemical screening of *Punica granatum* pomegranate showed that compounds like gamma-tocopherol showed antibacterial activity against many oral pathogens including *Staphylococcus aureus*, *Streptococcus mutans*, *Streptococcus salivarius*, *Staphylococcus epidermis*, *Lactobacillus acidophilus* and also antifungal activity against *Candida albicans* (Nord et al., 1972) Even the compound 9,12,15- octadecatrienoic acid isolated from *Aloe Vera* succulent, showed significant antibacterial and antifungal activity against *Staphylococcus aureus*, *Streptococcus pyogens*, *Pseudomonas aeruginosa* and *E.coli*. it showed inhibition of fungus such as *Aspergillus flavans* and *Aspergillus niger*. (Arunkumar and Muthuselvam, 2009). Sterols such as Clerosterol which also showed good binding energy with the *MexAB* efflux pump system has been reported to have in vitro antimicrobial activity against fungi, yeast, and bacteria using ethanol and aqueous extracts of *Cassia fistula*. It

also has been reported to show anti-inflammatory activity. (Patrícia et al., 2007)

A recent homology on the RND MexXY another overexpressed protein present in MDR *Pseudomonas aeruginosa* showed in silico screening with common antibiotics. The study showed that the efflux pump exhibited an interaction as follows Penicillin (T.E= -8.3J), Chloramphenicol (T.E= -6.7J), Lincomycin (T.E= -7.2J) and Tetracycline (T.E= -8.2). This shows that phytochemicals from natural sources act as extremely good efflux inhibitors than synthetic drugs on comparing their bonding energies (Vijaya et al., 2012). A review on the extrusion pump MexAB-OprM in *Pseudomonas aeruginosa* proposes that the pump itself could be used as a high throughput screening system for the designing of new novel antimicrobials (Kazuki, et al., 2015). On performing the Antibiogram, it was observed that the given strain showed resistance to Methicillin and Rifampicin. Targeting of the efflux pump mechanism as a possible reason for resistance shown by *Pseudomonas aeruginosa* using extracts of *Wrightia tinctoria* as a potential efflux pump inhibitor was executed (Mahendra et al., 2014). Accumulation assay, which was done to check the accumulation of the drug after blocking efflux pumps with the plant extract showed no accumulation. (Li et al., 1994) it could either be interpreted that the extract had no activity or else there is no active efflux pump mechanism of resistance. But, the tests done to check for antibacterial activity showed positive inhibitory effect and moreover *W.tinctoria* has also been reported to show positive EPI activity (Mahendra and Nityanand, 2009).

Hence, it could either possible that there was no active efflux pump in the isolated strain. Moreover, it is reported that an active

efflux pump mechanism in *P. aeruginosa* PA01, wild type, could naturally resist Aminoglycosides. A mutation in the genes has been reported to be the reason for the loss of the active efflux mechanism. (Julio et al., 1999; In-Kyoung et al., 2016) Our antibiogram has worked with Streptomycin which also confers no resistance being an Aminoglycoside. The study was therefore, taken to molecular precision. The target of the investigation now was to detect the presence of resistive genes. Polymerase Chain Reactions of the DNA from the isolated sample was done with SHV primers which showed amplification confirming the presence of the SHV gene sequence that relate to the ESBL family (Dirk et al., 2009). Resistance to drugs such as Methicillin and Oxacillin in *Staphylococcus aureus* is determined by the presence of *SCCmec*, a mobile genetic element present in their chromosomes which contains the *mecA* resistance gene. (John W. Pearman, Fred C. Tenover, et al, 2002) Rifampicin resistance in *M.tuberculosis* and *E.coli* has been reported to be due to mutation in the gene encoding for RNA polymerase subunit $\beta$  (*rpoB*) (Telenti et al, 1993) It is described as a “single step” high level resistance pattern that arises due to missense mutations in the 69-bp region of *rpoB*. (Kapur et al., 1994)

Resistance to drugs such as Methicillin and Oxacillin in *Staphylococcus aureus* is determined by the presence of *SCCmec*, a mobile genetic element present in their chromosomes which contains the *mecA* resistance gene. (Barben and Schmid, 2008) Rifampicin resistance in *M. tuberculosis* and *E. coli* has been reported to be due to mutation in the gene encoding for RNA polymerase subunit $\beta$  (*rpoB*). (Telenti et al, 1993) It is described as a “single step” high level resistance pattern that arises due to missense mutations in the 69-bp region of *rpoB* (Kapur et al., 1994). Modern quinolones such as Ciprofloxacin show increased activity and less frequency of being

resisted by bacterial resistance mechanisms. The target of these drugs is previously reported to be a subunit of the bacterial enzyme DNA gyrase and also another site in addition to this. It is also reported that this additional target decreases the susceptibility of the organism to Rifampin (Hooper et al., 1987). This phenomenon could also be a possible reason for the resistance shown by the *P. aeruginosa* under study against Rifampicin as seen in our Antibiogram. The study concludes that *Pseudomonas aeruginosa* that is responsible for most superficial infections has now developed resistance against many common antibiotics including Methicillin and Rifampicin. Presence of SHV genes being one of the reasons of resistance which is capable of showing activity like most ESBL organisms. Further study and molecular characterization of the strain is needed to find out any other potential mechanisms of resistance present in MDRPA and a cure from natural sources to fight against the crisis of Antibiotic Resistance in Nosocomial Infections.

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of the active efflux mechanism. Our antibiogram has worked with Streptomycin which also confers no resistance being an Aminoglycoside. The study was therefore, taken to molecular precision. The target of the investigation now was to detect the presence of resistive genes. Polymerase Chain Reactions of the DNA from the isolated sample was done with SHV primers which showed amplification confirming the presence of the SHV gene sequence that relate to the ESBL family (Dirk et al., 2010).

Resistance to drugs such as Methicillin and Oxacillin in *Staphylococcus aureus* is determined by the presence of *SCCmec*, a mobile genetic element present in their chromosomes which contains the *mecA* resistance gene. (Keiko et al., 2002) Rifampicin resistance in *M.tuberculosis* and *E.coli* has been reported to be due to mutation in the gene encoding for RNA polymerase subunit  $\beta$  (rpoB). It is described as a “single step” high level resistance pattern that arises due to missense mutations in the 69-bp region of rpoB (Kapur et al., 1994). Modern quinolones such as Ciprofloxacin show increased activity and less frequency of being resisted by bacterial resistance mechanisms. The target of these drugs is previously reported to be a subunit of the bacterial enzyme DNA gyrase and also another site in addition to this. It is also been reported that this additional target decreases the susceptibility of the organism to Rifampin (Hooper et al., 1987). This phenomenon could also be a possible reason for the resistance shown by the *P. aeruginosa* under study against Rifampicin as seen in our Antibiogram. Thus, from this study we can hypothesize and conclude that the extracts of *Wrightia tinctoria* possess such valuable phytochemical compounds might be used as an inhibitor alone or as a synergistic inhibitor with common antibiotics. However, further wet lab study and much more precise results are required in this area to validate the study.

## 5. Conclusion

The study concludes that *Pseudomonas aeruginosa* that is responsible for most superficial infections has now developed resistance against many common antibiotics including methicillin and rifampicin. Presence of SHV genes being one of the reasons of resistance which is capable of showing activity like most ESBL organisms. Further study and molecular characterization of the strain is needed to find out any other potential mechanisms of resistance present in MDRPA and a cure from natural sources to fight against the crisis of antibiotic resistance in nosocomial infections.

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**Conflicts of Interest:** None

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