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Inhibiting Gram Negative Bacterial Drug Efflux Pump by Combating Medicinal Plants: *Bianceae sappan*, *Indigofera aspalathoides* and *Swertia chirayita* by *in vitro and in silico analysis*

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Abstract

Resistance to fluoroquinolones mainly reveals the decrease in drug accumulation due to presence of efflux pumps. These efflux pumps are recognized as a key factors of Antimicrobial resistance (AMR). The drug resistant isolates are not receptive to diverse mode of action of antibiotics which are in treatment options. To combat the AMR, efflux pump inhibitors (EPIs) are essential. Therefore the necessity of having to potentiate the antibiotics along with plant compounds. Hence in our investigation, ethanolic extracts of *Bianceae sappan*, *Indigofera aspalathoides* and *Swertia chirayita* were used to screen the antibacterial activity and synergistic interaction with ciprofloxacin against fluoroquiolone resistant bacteria. It's quite essential to do *in silico* analysis to identify the effective inhibitor. Overall there are thirty two isolates which includes eighteen of *E.coli* and fourteen of *Klebsiella pneumoniae* collected from The Oxford Medical College, Hospital & Research Jigala, Bangalore, Karnataka, India. These isolates were screened for antimicrobial susceptibility test (AST), and found that 87% resistant to ciprofloxacin and norfloxacin. These resistant isolates were screened by MIC – ciprofloxacin and EtBr plate assay in order to check the existence of efflux pump. The increased MIC for ciprofloxacin and less amount of fluorescence suggested that, the presence of efflux pump in three *E.coli* (E1, E2 and E3) . In the synergistic studies, particular extract of *Bianceae sappan* showed reduction in MIC along with ciprofloxacin and followed by *Indigofera aspalathoides* and *Swertia chirayita* extracts. Further, in silico analysis were carried to scrutinize the effective compounds, as efflux pump inhibitor. The crystal structure of AcrB from PDB id : 5ENO, and 2W1B displayed better interaction and binding energy with ligands of ethanolic extract of *Bianceae sappan*, and followed by *Indigofera aspalathoides* and *Swertia chirayita*, as compared to control (PaβN). Thus, the plant based phytomolecules has promising role to inhibit the bacterial efflux pump.

Keywords: *Antibacterial activity, Minimum inhibitory concentration, Synergistic studies, Phenylalanine-arginine β Naphthylamide*

I. INTRODUCTION

Antibiotics, is a well-known antimicrobial agent for life threatening bacterial infections caused either by Gram negative or Gram positive Bacteria. The increased antimicrobial resistance is due to abuse of OTC antibiotics, inadequate usage of antibiotics and rigorous usage of animal husbandry. However the major spread

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of resistance gene in the bacterial species, is basically due to vertical or horizontal gene transfer mechanisms. These resistance genes might be harbouring in plasmid or chromosomal mediated and its characteristics might be recognized to the abilities of these resistant isolates in altering their genetic makeup very rapidly [1, 2] . Another, major concept for the multidrug resistance is the consequence of over expression of bacterial efflux system that expels the antibiotics that are in the present usage by preventing its appropriate mode of action. Most interestingly, all the bacterial species harbor efflux pumps, which encoded by either chromosomal or plasmid mediated function. These efflux pumps are classified into five families, which include resistance nodulation division RND family, the major facilitator superfamily family (MFS), the ATP (adenosine triphosphate) - binding cassette (ABC) superfamily, the small multidrug resistance (SMR) and the multidrug and toxic compound extrusion (MATE) family. Among them, RND pump is only found in Gram negative bacteria, other four pumps are extensively disturbed in both Gram negative and Gram positive bacteria. The RND pump has been associated with clinical significant antibiotic resistance, seen in *E.coli* and *K.pneunomiae* [3, 4]. Therefore, the antibiotics in regular use, new or combination is not able to control the infections effectively by the presence of efflux pump. In order to overcome the problem, efflux pump inhibitor (EPI's) which binds to the efflux pump and antibiotics are administrated together. There are many medicinal plants that have been used traditionally for various infections caused bacteria. The plant derived compounds possess major function as a potent antibacterial agent by the presence of secondary metabolite. These compounds might have direct antimicrobial effect or can interact with key function of pathogens, thereby decreasing the potentiality of bacteria to develop resistance [5,6]. The necessity of blocking the efflux pump is more essential to use the regular antibiotics in combination with EPI's and thus efficiency of antibiotics can be improved.

In our present investigation, ethanolic extracts of *Bianceae sappan, Indigofera aspalathoides* and *Swertia chirayita* were used to screen the antibacterial activity with the combination of ciprofloxacin against *E.coli* and *K.pneumoniae* and also analyse the bioactive compounds against the efflux pump by *in silico* method.

II. MATERIAL AND METHODS

Selection of Plant and Preparation of plant extracts

The stem of *Biancaea sappan*, the stem and root of *Indigofera aspalathoides* and the stem and leaves of *Swertia chirayata* were used for plant extract which were collected from GKVK, Bangalore, Karnataka [7]. This extracts stored at room temperature.

Bacterial isolates

Bacterial isolates were collected form The Oxford Medical College, Hospital and Research Jigini, Bangalore, Karnataka, India. Thirty two isolates of *E.coli* (18) and *Klebsiella pneumoniae* (14) were subcultures on BHI agar plates and identified by biochemical test and stored in semisolid LB agar.

Antimicrobial susceptibility test (AST)

AST were accomplished by conventional Kirby-Bauer disk diffusion method on Muller- Hinton Agar. In this test, control *E.coli* ATCC 25922 used as sensitive strain and along with isolates (*E.coli* and *K.p pneumoniae*) were screened for susceptibility for antibiotics such as Norfloxacin (10mcg), Ciprofloxacin (5mcg), Cefoxitin (30mcg) and Amoxyclav (30mcg) (Hi-Media). The results were interpreted as per the guidelines of CLSI [8].

Determination of MIC

The Minimal Inhibitory Concentration (MIC) ciprofloxacin hydrochloride and ethanolic plant extract was used as stock 1mg/ml and 150mg/ml of DMSO respectively [9]. The MIC for both antibiotic and ethanolic plant extract was determined for all the isolates by serial dilution from the 1st to 10th well using the 96 well plate method and resazurin dye This assay works on the concept of oxidation-reduction reaction, using resazurin dye (0.6%) [10]. The experiment was done in duplicates along with *E.coli* ATCC 25922 used as sensitive strain.

Cartwheel Method

This method is a agar-based method which exploits EtBr for the demonstration of efflux pump activity harboured in bacteria [11]. In this methodology, trypticase Soy Agar (TSA) plates containing EtBr concentration of 2.5mg/L were prepared in duplicates. The isolates were strike on the EtBr-TSA plates and incubated at 37°C for 16 hours. The results were interpreted by visualizing the plates under UV transilluminator, in order to find out the existence of efflux pump in bacterial isolates.

In vitro checkerboard synergy assay

The synergistic interactions were estimated by checkerboard method [12]. Concisely, a series of two fold dilutions of ciprofloxacin and plant extracts concentration from its MIC value to 1/32 MIC were furnished, antibiotic and the plant extract was mixed together. By this method, 36 different combinations of the antibiotic and plant extract was established for analysis. Each well confined unique combination of plant extract/antibiotic concentration in a volume of 200 µl and 10 µl of the bacterial suspension (0.5 McFarland Standard). The plate was incubated at 37°C for 24 h to

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48 h. To analyse the bacterial growth after incubation period, 10 µl of resazurin (0.62%) solution was added to each well and incubated at 37℃ for 2 hours. The colour change were observed and taken for calculation fractional inhibitory concentration index (FICI) [13].

FIC Index =

$$\frac{\text{MIC of Plant Extract in Combination}}{\text{MIC of Plant Extract alone}} + \frac{\text{MIC of Antibiotic in Combination}}{\text{MIC of Antibiotic alone}}$$

Interpretation of (FICI):

FICI = 0.5 = synergy (joint effect is greater than sum of individual activity). FICI > 0.5 to 4 = indifference (joint effect is equal to sum of individual activity). FICI > 4 = antagonism (joint effect is less than sum of individual activity or effect of individual activity).

Gas Chromatography – Mass Spectrometry Analysis

The JEOL GCMATE II GC-MS with data system is a high resolution, double focusing instrument. Maximum resolution: 6000 Maximum calibrated mass: 1500 Daltons. Source options: Electron impact (EI); Chemical ionization (CI), were carried in School of advanced Sciences VIT University, Vellore, Tamil Nadu.

In *Silico* Analysis

Three-dimensional molecular organization is one of the practicalities of structure-based drug design studies. From the program Auto dock 4.2.6 , automated docking of ligands to their macromolecular targets. Two receptors were taken from RCSB PDB, PDB id : 5ENO (MBX2319 bound structure of bacterial efflux pump) and 2W1B (AcrB in complex with bile acid). The following step was involved in the docking process, preparation of ligand and molecule and further run the auto grid and auto dock. Finally, the .pdb format file opened in the Discovery studio visualizer version 3.5 and the 3-D protein-ligand interactions and binding energy were analysed, as a control PaβN was used.

III. RESULT AND DISCUSSION

Fluoroquinolone resistance of isolates

Clinical isolates, *E.coli* and *Klebsiella pneumoniae* collected from The Oxford Medical College, Bangalore were analysed for their resistance pattern of fluoroquinolone antibiotics – ciprofloxacin and norfloxacin. Among the thirty two isolates, 18 of *E.coli* and 14 of *Klebsiella pneumoniae* showed 87% resistant to ciprofloxacin and norfloxacin. The antibiotic cefoxitin showed 100% resistant, as a control *E.coli* ATCC 25922 was used. However, for Amoxyclav 100% resistant were observed shown in figure 1. The major threat is fluoroquinolone resistant is higher and disquieting for the treatment.

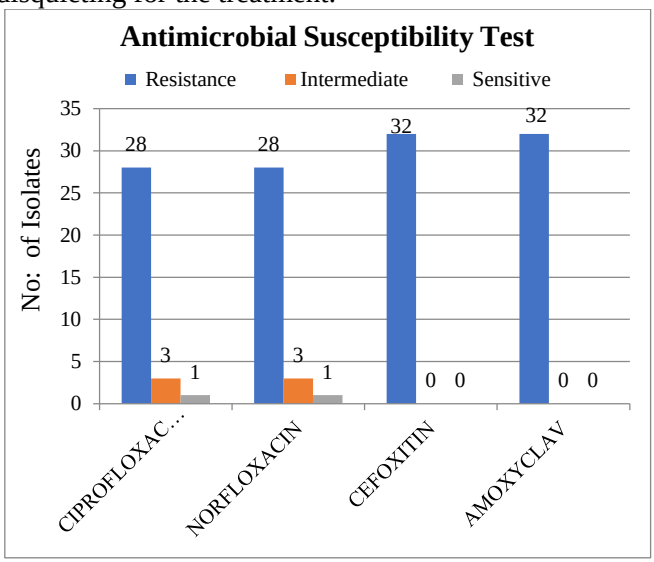

Fig 1 Antimicrobial Susceptibility test of clinical isolates

Association with MIC to Ciprofloxacin and presence efflux pump by Cart wheel method

The resistant isolates were analysed for the presence of efflux pump by EtBr assay. By comparing other isolates, only three of *E.coli* (E1, E2 and E3) resistant isolates showed no fluorescence when exposed to the UV transilluminator. This could be due to presence efflux pump, which eventually eliminate the EtBr rather pass through the pump into the cell. In order to correlate fluoroquinolone resistance, minimal inhibitory concentration of ciprofloxacin was determined. It was found that E1, E2 and E3 isolates exhibited MIC > 125 µg/ml, while all other isolates exhibited MIC > 62.5 µg/ml. However, *K.pneumoniae* isolates, does not show elevated MIC, which was ranged from < 31.25 to 15.62 µg/ml. As a control sensitive strain *E.coli* ATCC 25922 was used. From our result, the three *E.coli* (E1, E2 and E3) isolates disclosed high resistance for ciprofloxacin and also exhibited the presence of efflux pump. This was supported by a previous study, reported with upregulated CIP-R (ciproflaxocin resistance) *E.coli* isolates were shown with only acrB gene expression [14].

Antibacterial assay of plant extract

In this study, three resistant isolates were screened for MIC and synergistic activity using plant extracts and control sensitive strain was included. The ethanolic extract of *Biancaea sappan* (BSE), *Indigofera aspalathoides* (IAE), and *Swertia chirayita* (SCE) were used to screen the antibacterial activity, as shown in table 1. Analysing the MIC of the each extract, the highest range was observed in *Biancaea sappan* 18750 -

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75000 µg/mL followed by *Indigofera aspalathoides and Swertia chirayita* 37500 - >75000 µg/mL respectively, the results of MIC ciprofloxacin, plant extract and cart wheel method were represented in the table 1.

A similar study in Shahid Motahari Hospital, Tehran, 40 isolates had exhibited resistance to ciprofloxacin, tetracycline, ceftazidime and gentamicin which has expressed in increased levels of the AcrAB efflux pump mainly in ciprofloxacin resistant strains [15]. Another study on *E.coli*, an different mechanism seen in resistance to fluoroquinolones : such as mutations in genes encoding of various resistant factor and also associated with decrease in drug accumulation thus increases the bacterial impermeability or an active drug efflux [14].

Table 1 Results of Minimal Inhibitory concentration – Ciprofloxacin, plant extracts and cart wheel method

Isolate Code	CIP µg/ml	BSA µg/ml	IAE µg/ml	SCE µg/ml	EtBr-plate assay
E1	>125	18750	37500	37500	+
E2	>125	18750	37500	37500	+
E3	>125	18750	18750	18750	+
***E.coli* ATCC 25922**	S	1172	2344	2083	–

E1, E2 and E3 *– Fluoroquinolone (Ciprofloxacin and Norfloxacin) resistant E.coli isolates, CIP- Ciprofloxacin,* ***S****- Sensitive , '+' denotes presence of efflux pump , ' - ' denotes absence of efflux pump*

Presence of efflux pump does not alone expel a broad range of antibiotics, also drive the acquisition of additional resistance mechanisms by lowering the intracellular antibiotic concentration and promoting the mutation accumulation. Therefore, combination assay was carried out in order to find out whether the plant compound would be used as an effective EPI's.

Synergistic –Checkerboard assay

For the three fluoroquinolones resistant isolates, a synergistic consequence was observed between ciprofloxacin and plant extracts, such as ethanolic extract of *Biancaea sappan, Indigofera aspalathoides and Swertia chirayita.*. FICI of the combination of *B.sappan* and ciprofloxacin, and followed by other two extracts. The checkerboard assay revealed that plant extracts have synergistic effect with ciprofloxacin. It is possible to reduce the MIC of ciprofloxacin along with sub inhibitory concentration of plant extracts. Therefore, synergistic activity might be due to presence of bioactive efflux pump inhibitor (EPI) compound found in medicinal plant extract and thus it blocks the efflux pump effectively. Thus, inhibitor binds to the efflux pump possessing fluoroquinolone resistant isolates and expel of the antibiotics had immobilized. For this reason, we suggest that the checkerboard method is a more sensitive method for evaluating synergistic interaction with MIC value [16]. Antagonistic effect was not found in these combinations. Further, *in silico* analysis was carried out to find the better interaction and binding energy between the efflux pump crystal structures from PBD bank.

Table 2 FIC Index of the ethanolic extract of *Biancaea sappan, Indigofera aspalathoides* and *Swetia chirayata*

Plant name	FIC Index		
	E1	E2	E3
***Biancaea sappan* (BSA)**	0.37 (S)	0.18(S)	0.18(S)
***Indigofera aspalathoides* (IAE)**	0.26(S)	0.37(S)	0.75(I)
***Swertia chirayita* (SCE)**	0.18(S)	0.37(S)	1(I)

FICI – Fractional Inhibitory Concentration Index, S – Synergism, I – Indifference

GCMS Library Structure *and In-Silico* analysis

From the library output of GCMS, ligands were identified using PubChem and listed in the table 3.The GCMS chromatography of the ethanolic plant extracts as shown in figure 2a, 2b and 2c.

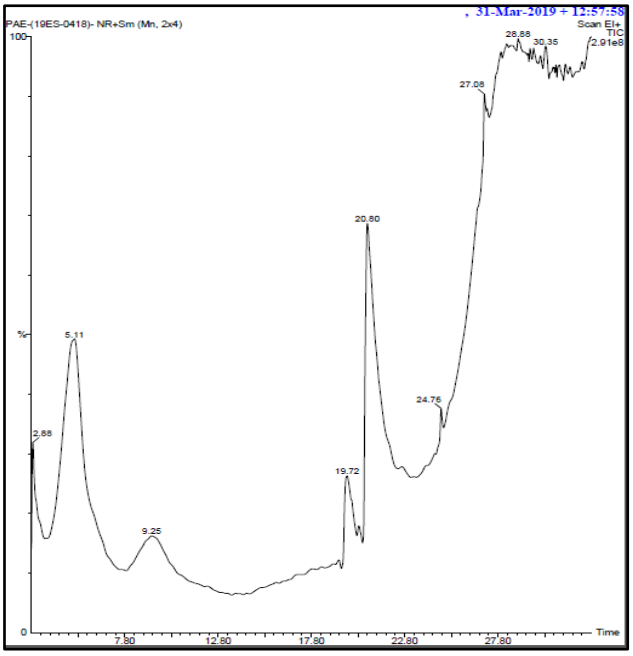

Figure 2a: Represents the GCMS peak (Chromatogram) of *Biancaea sappan*

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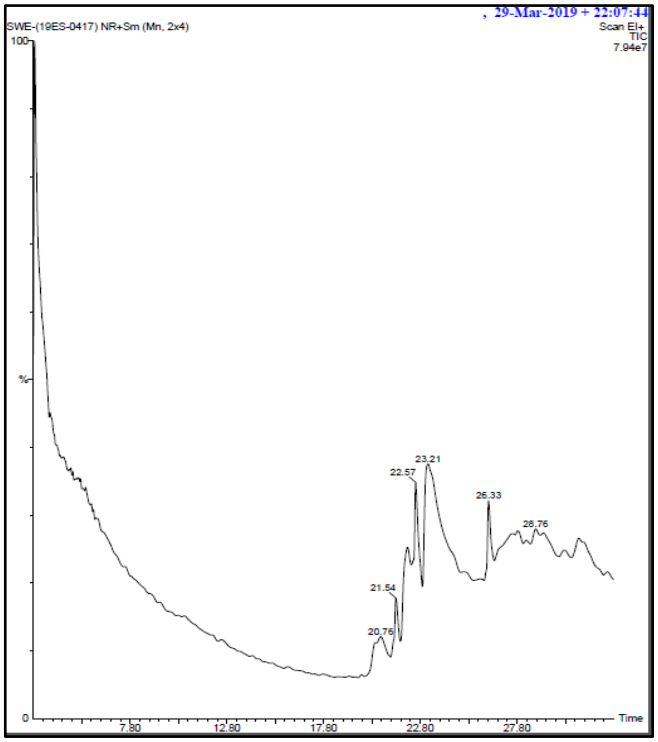


Figure 2b: Represents the GCMS peak (Chromatogram) of *Indigofera aspalathoides*

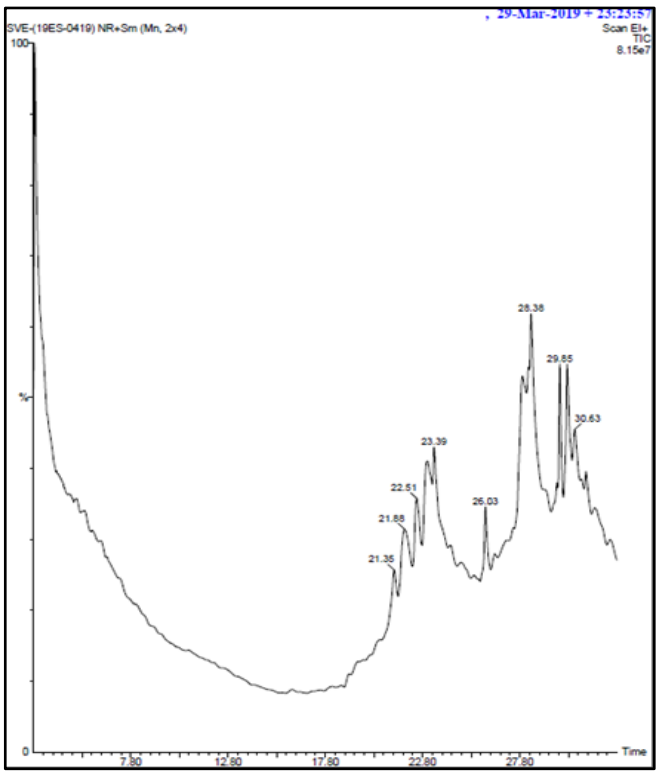
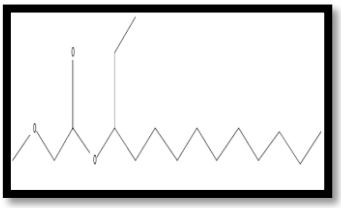
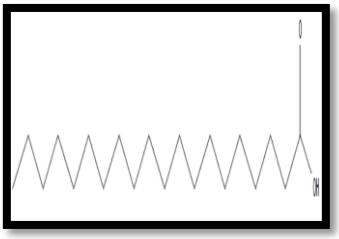
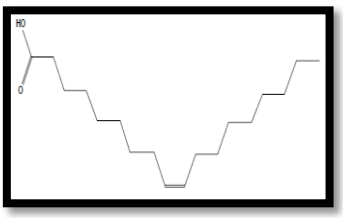
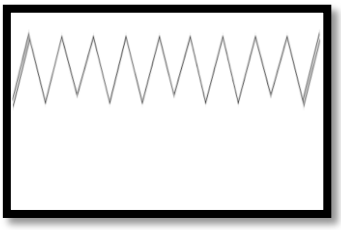


Figure 2c: Represents the GCMS peak (Chromatogram) of *Swetia chirayata*

Docking evaluation for the best docked ligands from the ethanolic extract of *Biancaea sappan, Indigofera aspalathoides* and *Swetia chirayata*

The Gram negative isolates, particularly enteric pathogens *E.coli* and *K.pneumoniae* possess tripartite AcrAB-TolC, a RND based efflux pump. This system is a tripartite complex, comprised of the periplasmic accessory protein, Acr A mediates the interaction between AcrB and TolC. AcrB transports drugs by functionally rotating process in which the periplasmic domain of each protomer adopts exclusive conformations called contact, binding and finally expel the substrates [17]. Hence, AcrB receptor is taken for the *in silico* analysis from the PDB id: 5ENO, 2W1B and as control Phenylalanine-arginine beta-naphthylamide (PaβN) was used.

Table 3. Library output of GCMS ligands from the ethanolic extract of *Biancaea sappan, Indigofera aspalathoides* and *Swetia chirayata*

Name of the Plant	Compound (Ligand)	Structure
Biancaea sappan	Methoxyacetic Acid, 3-Tridecyl Ester	
	Eicosanoic Acid	
	Oleic Acid	
	1,19-Eicosadiene	

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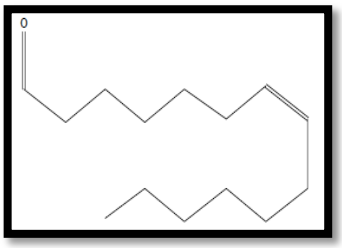
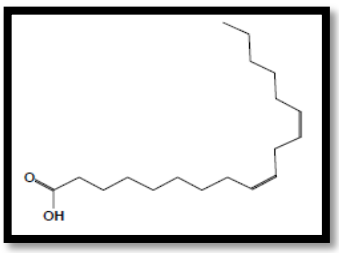
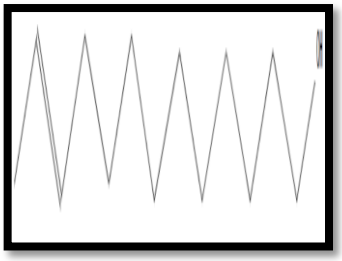
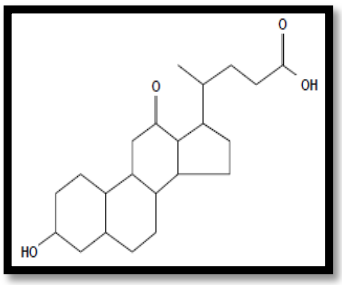
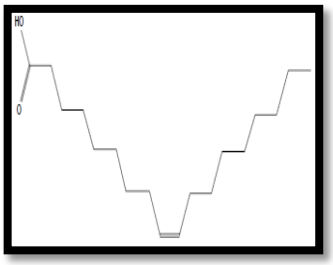
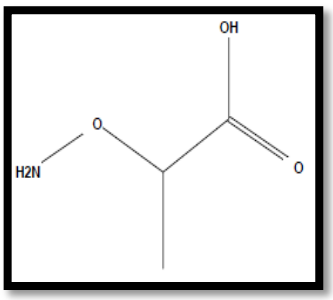
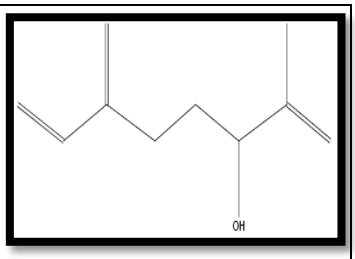
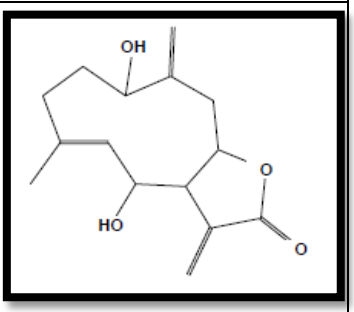
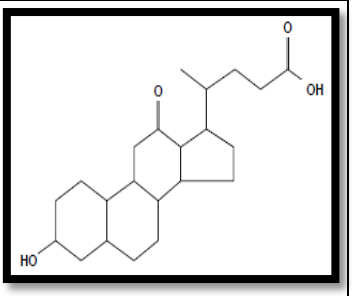
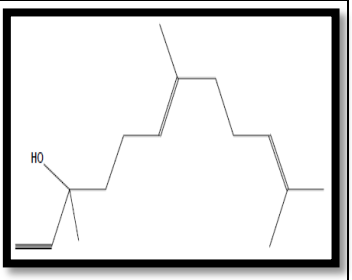
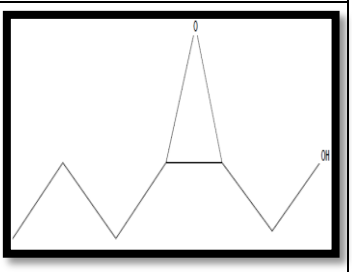
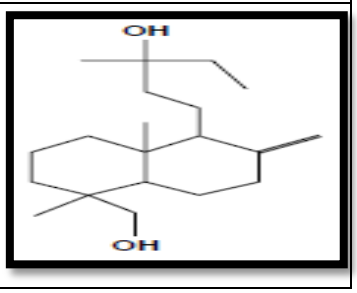
	7-Tetradecenal, (Z)-	
	9,12-Octadecadienoic Acid (Z,Z)-	
	11-Tridecen-1-Ol	
	3-Hydroxy-12-Ketobisnorcholanic Acid	
	Oleic Acid	
	Propanoic Acid, 2-(Aminooxy)-	

Indigofera aspalathoides	2-Methyl-6-Methylene-Octa-1,7-Dien-3-Ol	
	7oxabicyclo[4.1.0]Heptane,1 Methyl-4-(2-Methyloxiranyl)-	
	3-Hydroxy-12-Ketobisnorcholanic Acid	
	6,10-Dodecadien-1-Yn-3-Ol, 3,7,11-Trimethyl	
	(2s,3s)-(-)-3-Propyloxiranem ethanol	
	1-Naphthalene propanol, .Alpha.-Ethyldecahydro-5-(Hydroxymethyl)-.	

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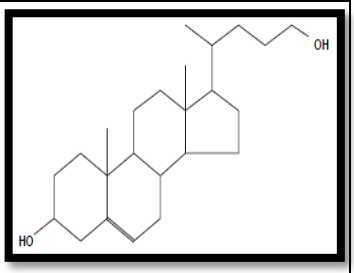
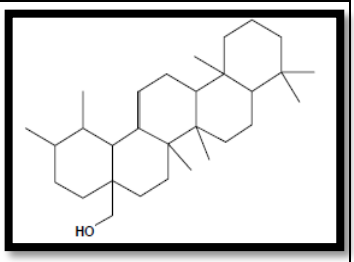
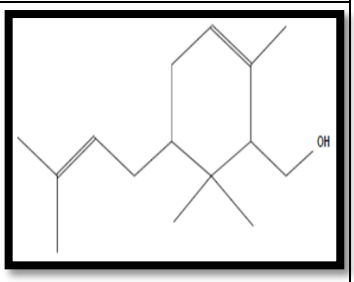
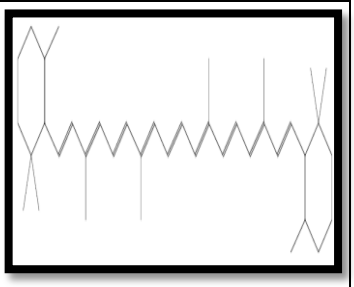
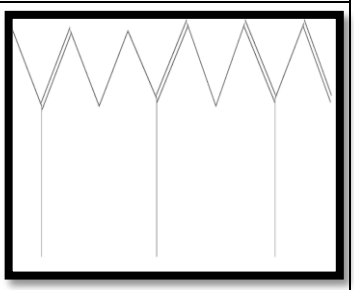
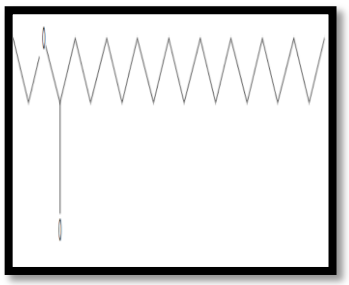
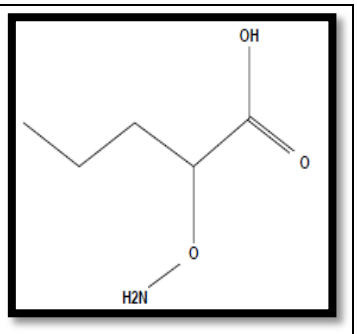
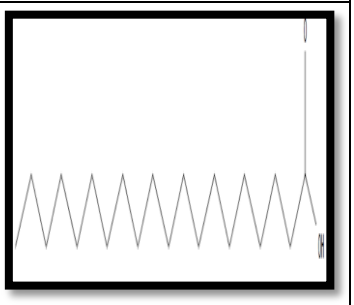
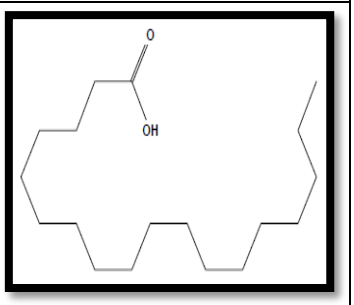
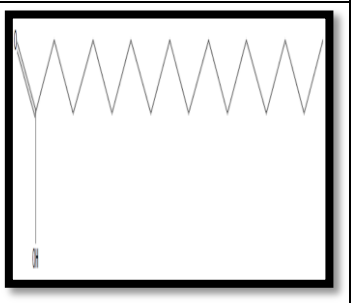
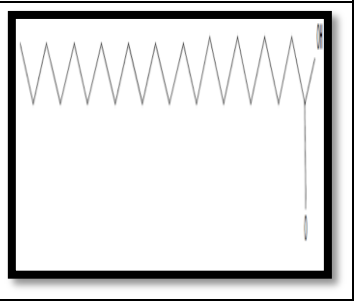
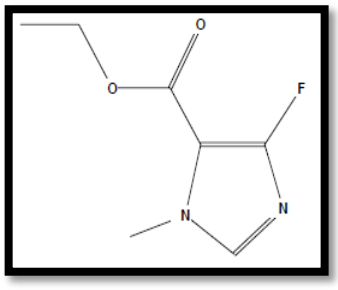
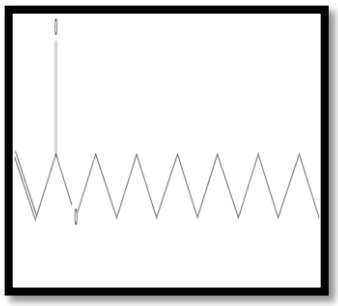
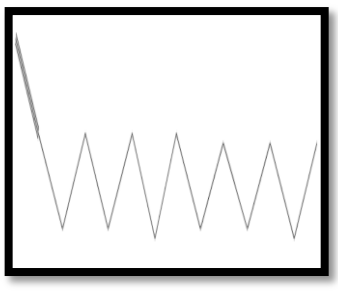
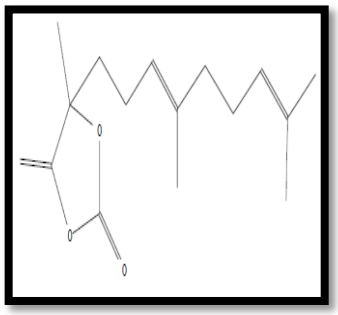
	5-Cholene, 3,24-Dihydroxy	
	Urs-12-En-28-Ol	
	2,4,4-Trimethyl-3-Hydroxymethyl-5a-(3-Methyl-But-2-Enyl)-Cyclohexene	
	Beta. Carotene	
	Alpha.-Farnesene	
	Octadecanoic Acid, Ethyl Ester	
Swetia chirayata	Pentanoic Acid, 2-(Aminooxy)-	
	Eicosanoic Acid	
	Octadecanoic Acid	
	N-Hexadecanoic Acid	
	Docosanoic Acid	

	4-Fluoro-1-Methyl-5-Carboxylic Acid,Ethyl(Ester)	
	2-Propenoic Acid, Oxybis(Methyl-2,1-Ethanediyl) Ester	
	1-Tetradecyne	
	1,3-Dioxolan-2-One, 3-Methyl-3-(4,8-Dimethylnona-3,7-Dienyl)-4-Methylene	

Docking were performed to find the interaction of the ligands in the active sites, thus provides information about the inhibitory action. The better interactions and affinity binding energy were detected with four ligands. For the results of computational studies the ligands, Oleic acid, 3-Hydroxy-12-Ketobisnorcholanic Acid and 1,3-Dioxolan-2-One,3-Methyl-3-(4,8-Dimethylnona-3,7-Dienyl)-4-Methylene has given better binding energy of -4.73, -3.65 and -4.47, which is comparable to control PaβN the docking score – 3.78 when docked with crystal structure of AcrB, PDB id : 5ENO Similarly, other two more ligands, Urs-12-En-28-Ol and 1,3-Dioxolan-2-One,3-Methyl-3-(4,8-Dimethylnona-3,7-Dienyl)-4-Methylene binding energy of -5.72 and -3.31, which is compared with control PaβN – 2.47, when docked with the crystal structure of AcrB, PDB id : 2W1B. The interaction studies were listed in the table 4.

The interaction studies revealed that the ligands were interacting with the active site residues of crystals similar as in the control. The interactions are covalent and non-covalent such as hydrogen bond and Pi- sigma, Pi-Pi, Pi- alkyl, alkyl was distinguished with ligands shown in figure 3, 4,5,6, and 7 compared with control shown in figure 8 and 9. Both the bondings are involved in the key interactions which reveal the activity and its dynamic behaviour observed in four ligands.

Therefore, the docking results of *in silico* data validated by the interactions and showed specificity towards blocking the efflux pump crystal structure in order increase the efficiency of antibiotics. The above results also been supported with *in vitro* validation by MIC and combination assay, with the ethanolic extract of *Biancaea sappan* followed by *Indigofera aspalathoides* and *Swetia chirayata*.

Table 4. *In- Silico* docking analysis of ligand interactions with crystal structure of PDB id :AcrB, 5ENO and 2W1B

Ligands and Control	Efflux pump receptor	Interaction	Residues	Binding Energy (kcal/mol)
C1	5ENO	Hydrophobic	PRO B116, MET A115	-4.73
C2	5ENO	Hydrophobic	PHE C178 VAL C139, PHE C628, ILE C277, VAL C612, ALA C279 , PHE C610	-3.65
C3	5 ENO	Hydrophobic	PHE C 628 , PHE C 615 VAL C139, PHE C178, ILE C277, PHE C617, PHE C610, VAL C612	-4.47
	2W1B	Hydrogen bond,	SER A 715, GLN C830	-3.31

		Hydrophobic	PHE A 64, PHE A666, ARG A 717 LEU A828	
C4	2W1B	Hydrogen bond,	ASN A719	-5.72
		Vander waal interaction	SER 715, LEU A828, GLY A270, PRO A718, THR A652	
		Hydrophobic	ARG A717 PHE A664	
PaβN	5ENO	Hydrophobic	PHE C178 ILE C277 VAL C139, AlA C279 ILE C277	-3.78
	2W1B	Hydrogen bond	SER A715	-2.47
		Hydrophobic	PHE A664 ARG A717	

Ligands are represented as - Oleic acid - C1 ; 3-Hydroxy-12-Ketobisnorcholanic Acid – C2 ; 1,3-Dioxolan-2-One, 3-Methyl-3-(4,8-Dimethylnona-3,7-Dienyl)-4-Methylene – C3; Urs-12-En-28-Ol - C4

From our investigation, the presence of potent efflux pump inhibitor intensifies these ethanolic extract of *Biancaea sappan, Indigofera aspalathoides* and *Swetia chirayata* may block the efflux pump effectively and use routine antibiotics to the controlling bacterial infections [18]. In the case of PaβN, a potent inhibitor which blocks the efflux pump effectively, studies showed that due to phototoxicity it cannot used in clinical purpose. However, further confirmatory studies will be required to confirm the appropriate role of ligands as a potent inhibitor that inhibits the bacterial efflux pump.

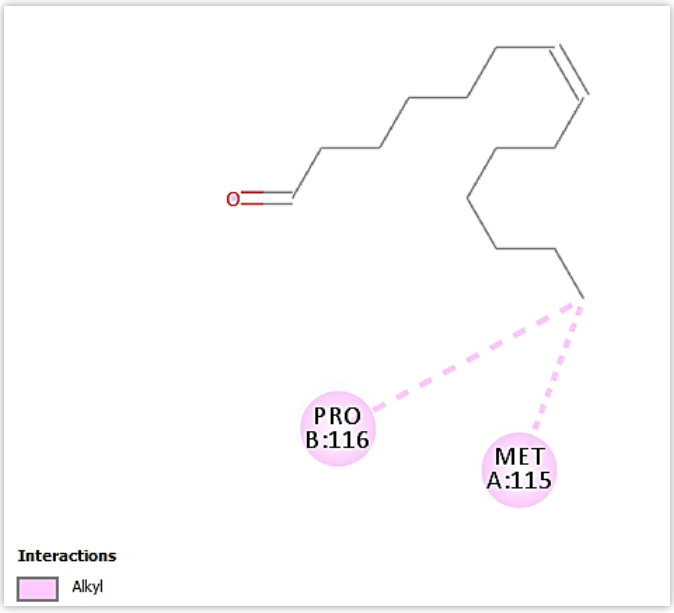


Fig 3 Interaction between 5ENO and Oleic acid

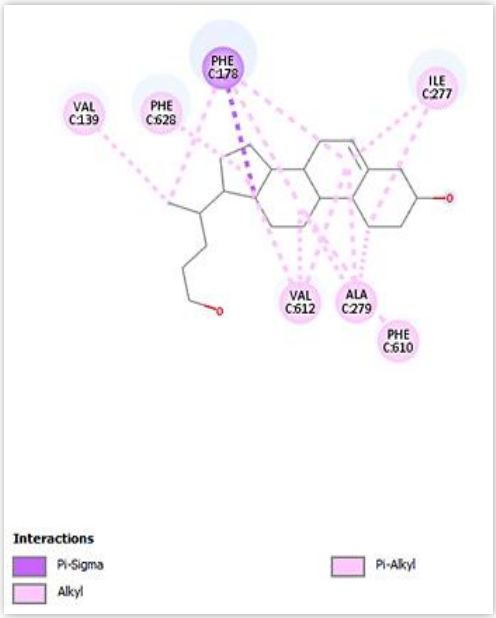


Fig 4 Interaction between 5ENO and 3-hydroxy- 12-ketobisnorcholanic acid

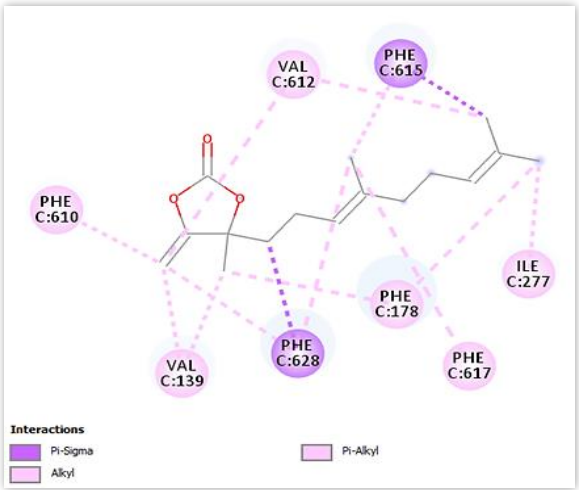


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Fig 5 Interaction between 5ENO and,3- Dioxolan-2-One,3-Methyl-3-(4,8- Dimethylnona-3,7-Dienyl)-4-Methylene

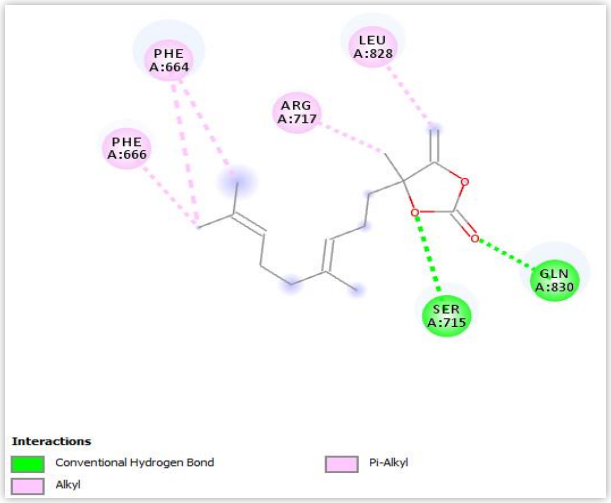

Fig 6 Interaction between 2W1B and 1,3-Dioxolan-2-One,3-Methyl-3-(4,8-Dimethylnona-3,7-Dienyl)-4-Methylene

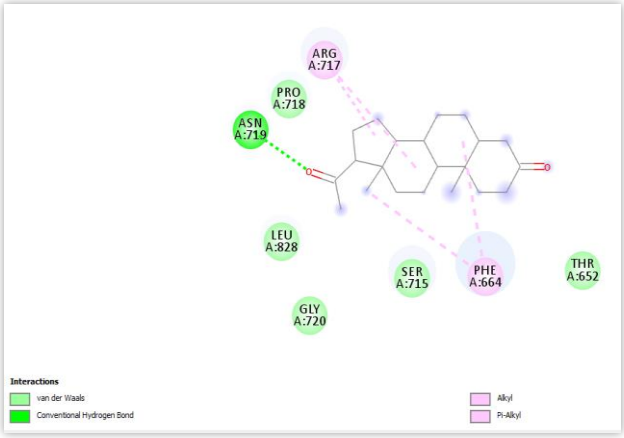

Fig 7 Interaction between 2W1B and URS-12-en-8-ol

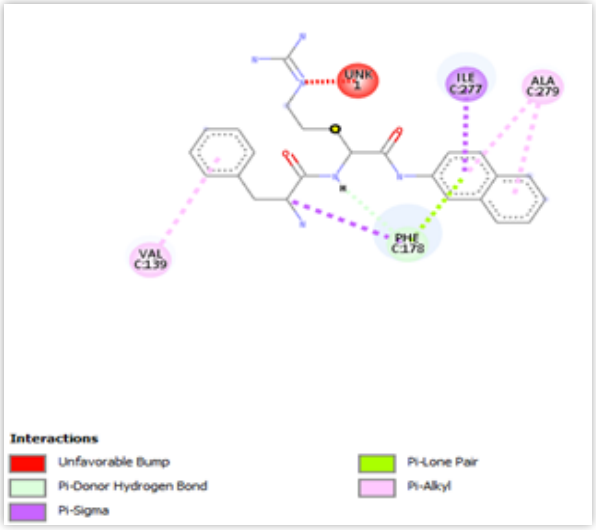

Fig 8 Interaction between 5ENO and PaβN

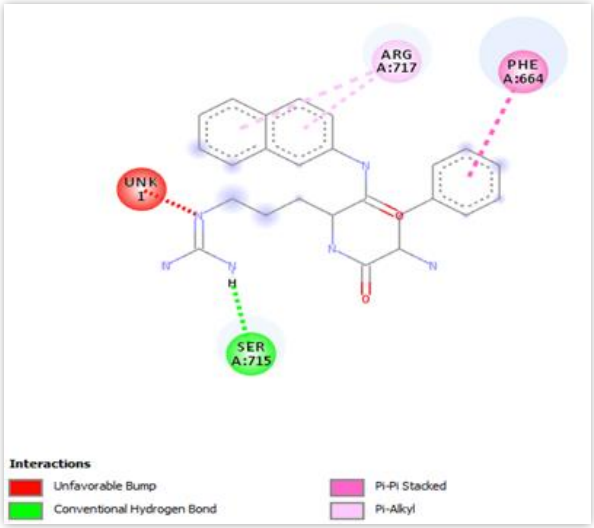

Fig 9 Interaction between 2W1B and Phenylalanine-arginine β Naphthylamide

IV. CONCLUSION

Hence, we would like to conclude that there is an effective synergistic interaction between the effective bioactive compounds (ligands) of the ethanolic extract of *Biancaea sappan, Indigofera aspalathoides* and *Swetia chirayata* in connotation with antibiotic, ciprofloxacin. Thus, this therapy with plant compounds and antibiotics have a great scope towards the infection control measures. Further studies are required in this field as there is a potential for antibiotics that are not in use due to development of resistance in bacteria by the efflux mechanisms, which need to be brought back into the market. Hence there might be no need for combination or newer antibiotics in the treatment along with EPI's.

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