Unveiling the role of phytochemicals in abating Antimicrobial Resistance in *Staphylococcus aureus*

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***Abstract*- On a global scale antimicrobial resistance (AMR) represents a significant menace to public health. *Staphylococcus aureus* is a member of the ESKAPE pathogens group which are of special concern owing to the rapid development of resistance. This has been correlated with a few mechanisms, one such being the expression of efflux pumps on the cell surface. The efflux of Antibiotics via bacterial MDR efflux pumps contributes to increased AMR prevalence heightening the need to counter this resistance mechanism by the discovery of novel therapeutics like efflux pump inhibitors (EPIs) that target the MDR (Multiple Drug Resistance) efflux pumps that are overexpressed to reduce antibiotic efficacy. Natural phytomolecules such as Piperine (PIP) an alkaloid found in Piperaceae family and Limonene (LMN) a terpene found in Rutaceae family show promise as novel natural EPIs by potentiating the activity of antibiotics. The present work aims to demonstrate the synergy and increase in efficacy (inhibition) of Tetracycline when coupled with these phytomolecules at their therapeutic concentrations. Further through an Ethidium Bromide Efflux Assay the role of these phytomolecules as EPI's is also explored. A significant fold reduction in bacterial viability was observed indicating that phytomolecules potentiate the effect of the antibiotic. In silico docking further validated that the phytochemical piperine, demonstrated higher binding affinity with some known efflux pumps, such as NorA and ABC transporter proteins. The study indicates that augmented inhibition can be achieved at the same dosage or lower, translating to the use of lower therapeutic concentrations of antibiotics for treatments of these infections when supplemented with phytomolecules, a crucial step in combating the rise of AMR.**

Index Terms*: AMR, Efflux Pump Inhibitors, Phytomolecules, *Staphylococcus aureus

I. INTRODUCTION

1.1 Antimicrobial resistance (AMR)

AMR is the elimination of sensitive bacteria through natural selection that results in the emergence of resistant bacteria within surviving populations. Amendments to the policies regulating the use of antibiotics and other antimicrobial agents have led to the emergence of antimicrobial resistance. The evolution of new bacterial strains due to selective pressure and the rampant misuse of antimicrobial agents are therefore resulting in an increasing rapid rate of antimicrobial resistance around the world. According to The World Health Organization (WHO), there has been an increase in antimicrobial resistance among the ESKAPE pathogens, which is listed among the WHO top 10 global concerns in public health. These infections require patients to undergo long term follow up, hospital admissions, multiple medications all of which have multiple complications and comorbidities hence posing great economic burden to the patient as well as the healthcare institutions. These costs are due to the elaborate precautions required to confine and disinfect samples, isolates, and equipment that are in contact with such patients to prevent cross transmission, emergence and spread of new resistant strains to other personnel or other patients.

1.2 Global Burden of AMR

The rise in the number of AMR episodes is estimated to have contributed to 4.95 million excess mortality deaths in 2019 (Msemburi et al., 2023). Among these deaths, 1.27 million were directly attributable to antimicrobial-resistant pathogens and the death caused by AMR was higher than combined deaths by HIV/AIDS and malaria in that year. The majority (80%) of these deaths can be attributed to the growing resistance among ESKAPE pathogens, which include six specific bacterial species: *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa and Enterobacter sp*. Further it is estimated that AMR will contribute to 10 million deaths annually by 2050 and cost around 1 trillion US dollars in public health

infrastructure (Murray et al., 2022; Msemburi et al., 2023).

However, there is insufficient identification of novel natural antimicrobials and the synthesis of novel artificial ones because of research, regulation and associated costs. Additionally, the overall process of devising and synthesizing new antimicrobials, and then finding ways of scaling up production is a time-consuming process (Abushaheen et al., 2020). Furthermore, the synthesized antimicrobial compounds can become ineffective through various methods, including modifications to the binding sites of the compounds, conformational changes, or the compound may be removed from the cell. Thus, research in the production of drugs is orientated towards finding more economically favorable drugs like those for preventing chronic and treatable diseases – that guarantee higher revenues for a longer term. Also, the introduction of new Antimicrobials is likely to be tightly regulated due to the increasing problem of resistance and the time for which they are prescribed is vastly shorter than that for chronic diseases (Piddock, 2012) (Davies & Davies, 2010).

1.3 Resistance in *S. aureus*

S. aureus is a Gram positive facultative aerobic coccus, commonly associated with upper respiratory tract and skin Infections. It is estimated that 30% (3 in 10 people) of the human population carries the bacteria commensally in their nasal cavity or skin, asymptomatically, this also poses a greater risk of possible future infections in these individuals. (Chambers & DeLeo, 2009). The danger with such a large percent of the population carrying opportunistic pathogenic bacteria is that it allows for easy exchange of genetic information commonly via horizontal gene transfer that contributes to the spread and evolution of resistant strains. Further, there is an increasing prevalence of community-acquired strains of *S. aureus* that not only account for the majority of the resistant infections but are also found to be more Virulent (Chambers & DeLeo, 2009). Natural *S. aureus* is susceptible to every antibiotic ever developed (Chambers & DeLeo, 2009). The Increasing Global prevalence of Methicillin resistant *S. aureus* (MRSA) and strains of Vancomycin resistant *S. aureus* (VRSA), the latter of which is used as a last-line antibiotic against pathogenic *S. aureus* strains is of global concern.

Common antibiotics like Trimethoprim-Sulfamethoxazole and long acting Tetracyclines are increasingly being prescribed for soft tissue and skin infections of MSSA (Methicillin susceptible *S. aureus*) and MRSA (Pantosti et al., 2007). Tetracycline exhibits a bacteriostatic effect by binding to the aminoacyl site (A-site) on the 30S bacterial ribosome subunit, preventing the aminoacyl tRNA binding to the ribosome during translation preventing the further addition of amino acids and polypeptide elongation. Interestingly both sets of antibiotics work intracellularly and thus are susceptible to being targets of Efflux Pumps. Resistance to tetracycline in *S. aureus* is mediated by two different mechanisms that coexist within the same microbe active efflux of the drug by plasmid encoded genes (Tet K, Tet L) and by ribosomal protection proteins that inhibit Tetracycline binding to the Ribosome (Pantosti et al., 2007). Emergence of antibiotic resistance mediated by efflux pumps has been found to favor antibiotic classes like Tetracyclines, Fluoroquinolones and Macrolides, all of which are widely used in the treatment of infections (Costa et al., 2019). Hence it is of crucial importance to develop mechanisms to ensure the susceptibility of bacteria to these drugs.

1.4 Role of Efflux Pumps in Resistance

The two broad methods of acquiring resistance are by decreasing the affinity for the target site with the antibiotic or to reduce the active concentration of antibiotic within the cell (Blanco et al., 2016). The mechanism behind efflux in contributing to antimicrobial resistance was first described in *E.coli* with the discovery of tetracycline resistance genes allowing for the active efflux of tetracycline from the bacterial spp. (Murray et al., 2022). However, the evolution of bacterial efflux pumps to shuttle out not only antimicrobials but also heavy metals, organic pollutants, quorum sensing signals and bacterial metabolites significantly predates the discovery, production and use of antimicrobials by humans. (Blanco et al., 2016) (Van Bambeke et al., 2000) Bacterial bio-membranes are easily traversed by amphiphilic substances that pass the hydrophobic and hydrophilic regions of the phospholipid bilayer. Antimicrobial drugs by selection and design are also commonly amphiphilic and have a wide range of biological activities due to this characteristic (Van Bambeke et al., 2000). In bacteria alongside metabolic

functions, efflux pumps (EPs) are key regulators of toxins and drugs. Many bacteria have either chromosomally encoded on plasmid acquired multidrug or specific efflux pumps. This makes EPs an indispensable bacterial defense system and one of the leading mechanisms contributing to antimicrobial resistance.

EPs are characterized based on their energy source, phylogenetic relationship and substrate specificity (Van Bambeke et al., 2000). They are broadly divided into two categories based on their transport - primary active EPs and secondary EPs. The former use Adenosine triphosphate (ATP) as the primary energy source to exchange molecules from the cell with the environment, these belong to the ABC (ATP Binding Cassette) superfamily. The latter transports efflux compounds through symports, Antiports or proton exchanges by coupling efflux to downhill transport of an ion or other molecules along a concentration gradient. (Van Bambeke et al., 2000) These more or less belong to 4 superfamilies of EPs. The major facilitator superfamily (MFS), resistance nodulation division superfamily (RND), small multidrug resistance (SMR) and the multidrug and toxic compound extrusion (MATE) superfamily (Van Bambeke et al., 2000). Generally, even varied strains belonging to the same bacterial species exhibit the common chromosomally encoded efflux pumps.

EPs that aid in antibiotic resistance in *S. aureus* primarily belong to the MFS. They are composed of 12-14 transmembrane helices with 2 domains each with around 6 helices that form a transmembrane channel. Extrusion of intracellular substrates occurs through the antiport system using proton motive force (PMF) where the efflux of the substrate is coupled with energy generated by the movement of protons down their concentration gradient (Costa et al., 2013; Pantosti et al., 2007). Most of these Multi-Drug EPs and are responsible for the efflux of various substrates. In *S. aureus* NorA, NorB and NorC efflux pumps which are chromosomally encoded and commonly show a constant basal level of expression to prevent accumulation of their substrates intracellularly (Pantosti et al., 2007). NorA is MFS multidrug efflux pump involved in the export of hydrophilic fluoroquinolones, Quaternary Ammonium Compounds (QAC's) and dyes (like Ethidium bromide). It shares 44% homology with the MDR efflux pump Bmr in *Bacillus subtilis* and 24% homology with TetA, a Tetracycline specific efflux pump commonly found in enteric bacteria (Costa et al., 2013). Most *S. aureus* strains show a transient low basal level of NorA expression which accounts for some degree of reduced intracellular accumulation for their substrate compounds. However, due to positive selection, mutations in its promoter region or increased availability of substrates it can be constitutively expressed (Costa et al., 2013). Similarly, NorB is a structurally similar MFS MDR efflux pump that specializes in the export of hydrophobic fluoroquinolones and Tetracyclines in addition to all NorA substrates. It has also been implicated in the bacterium's response to acid shock (Costa et al., 2019). Both these efflux pumps along with various other resistance genes, virulence factors and metabolic processes like autolysis are controlled and regulated by the pleiotropic MrgA locus by an oxidation sensing mechanism (Costa et al., 2019). While antimicrobial resistance in *S. aureus* is primarily mediated by the Nor efflux pumps, various other plasmid encoded MDR efflux pumps can be acquired by the bacteria and transiently expressed in the presence of their substrates (Costa et al., 2013). Thus, inhibiting this group of EPs is crucial to maintaining the efficacy of intracellular antibiotics.

1.5 Phytomolecules as Efflux Pump Inhibitors

For centuries predating the discovery of antibiotics, humans have used plant extracts and derivatives for their medicinal and therapeutic purposes (Ranjan et al., 2022). However the mode of action and mechanism of most still remains relatively unknown thus a vast reservoir of diverse molecules remains unexplored for novel therapeutics (Ranjan et al., 2022). Most phytomolecules are secondary metabolites in their host

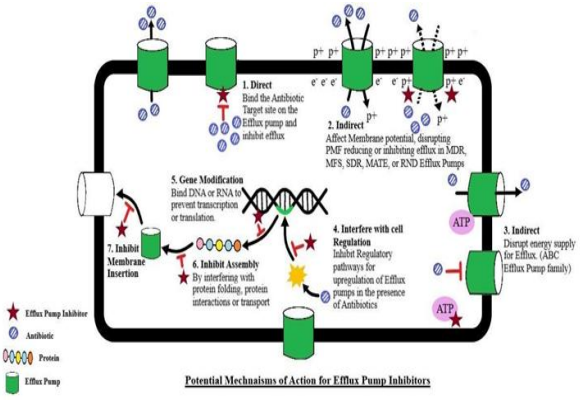

Fig.1 Potential Mechanisms of Action for EPI's.

plants, most commonly alkaloids, phenolics, sulfur compounds and terpenes. Many similar synthetic and microbial homologues are known antimicrobials or therapeutics. In the current age of rampant development of resistance, it is essential to "Rediscover" these therapeutics and understand their mechanisms and potential applications. These molecules can serve as Bioenchancers or potentiators of existing therapeutics even without any direct antimicrobial activity of their own but by working synergistically with exiting antimicrobials (Ranjan et al., 2022).

Recently synthetic drugs compounds are being used as EPIs, however the precise mechanism of action is poorly understood. Broadly with the available literature the five different strategies have been studied towards understanding the pathway inhibition. These include down regulation of efflux pump genes along with redesigning antibiotics that bypass substrate recognition by EPs, direct inhibition of functional pump assembly and blocking substrate entry into the active site as well as collapsing the energy mechanism responsible for ATP hydrolysis.

Thus, using a combination of conventional antibiotic and EPIs will result in an augmenting activity against bacteria that express efflux pumps. (Sharma, 2019) Thus, EPIs can be used as adjuvants in combination with convention antibiotics to enhance their activity against bacteria expressing efflux pumps (Sharma, 2019). Potential mechanisms of action for the Phytomolecules to function as EPI's as explored in Fig. 1

1.5.1 Piperine

Piperine (1-[5-[1,3-benzodioxol-5-yl]-1-oxo-2,4-pentadienyl]piperidine) extracted from the Piperaceae family is a type of amide alkaloid. It exhibits pleiotropic properties like antioxidant, anticancer, anti-inflammatory, antihypertensive, hepatoprotective, neuroprotective and enhancing bioavailability and fertility-related activities. Piperine has the ability to alter gastrointestinal disorders, drug-metabolizing enzymes, and bioavailability of several drugs (Haq et al., 2021). The molecular basis for the pleiotropic activities of piperine is based on its ability to regulate multiple signaling molecules such as cell cycle proteins, anti-apoptotic proteins, P-glycoprotein, cytochrome P450 3A4, multidrug resistance protein 1, nuclear factor-κB, MicroRNA, and antiviral activity against coronaviruses. Piperine also regulates multiple signaling pathways like JNK/P38 and MAPK (Haq et al., 2021). Additionally, Piperine does not undergo any metabolic transformations upon absorption (Haq et al., 2021). Further regular consumption of Piperine does not have any known toxic side effects. Sub-acute toxicity test in mice showed that Piperine is non-toxic up to a dosage of 100mg/kg of body weight (Haq et al., 2021). Piperine exhibits extensive immunomodulatory behavior protecting the Immune system during bacterial infections. These qualities make piperine an ideal therapeutic. Piperine has been proven to be a promising efflux pump inhibitor in bacterial MDR EP including the NorA EP in *S. aureus* (Kumar et al., 2008).

1.5.2 Limonene

Limonene (1-methyl-4-(1-methylethenyl)) is one of the most abundantly found terpenes in nature (Han et al., 2020). It has been shown to have several applications in food preservation and antimicrobial activity due to broad spectrum bactericidal activity, relatively low toxicity and its overall safety in consumption. D-Limonene prevents the growth of spoilage bacteria like *S. aureus*, *E. coli*, *P. aeruginosa* and *A. niger* among others along with several other Gram positive and Gram negative bacteria and certain fungi (Han et al., 2020). Increasing Limonene concentrations within bacterial cells show an upward trend in the conductivity of the bacterial cell membrane potential. Prolonged Limonene exposure in bacteria also shows loss of enzyme activity in enzymes like ATPases and inhibited metabolic activity of ATP production which also contributes to cell death (Han et al., 2020). Limonene was also found to have potential as an EPI against *S. aureus* contributing to reduced resistance. (de Araújo et al., 2021)

Further the role of both these phytomolecules will be explored as potential EPI's and potentiators of antimicrobial activity of Tetracycline against *S. aureus*.

II. MATERIAL AND METHODS

Bacterial Inoculum: The *S. aureus* culture was streaked on nutrient agar slants and incubated at 37°C for 24 hours. Then diluted in sterile saline to obtain an optical density (OD) of 0.5 at 540 nm. Total 50ul of this solution was used as a standard inoculum in 2ml for all assays. All experiments were carried out in triplicates.

2.1 Determination of Minimum Inhibitor Concentration (MIC):

Laboratory Culture of *S. aureus* was used to conduct a preliminary antibiotic sensitivity Tests using both agar cup diffusion and disk diffusion method on Tetracycline, Vancomycin, Erythromycin, Rifampicin and Ciprofloxacin.

Tetracycline stock solution was prepared from a commercial tablet, Resteclin (Tetracycline hydrochloride). MIC against tetracycline was determined by the Microbroth dilution method. The plates were inoculated with 5ul of the standard inoculum and incubated. Inhibition was estimated based on turbidity using an Aligent BioTek EPOCH Microplate Spectrometer, California, USA and later confirmed by spread plating to determine the colony forming unit (CFU).

2.2 Determination of Sub Inhibitory Concentration (SIC) of Phytomolecules:

Limonene (D-Limonene, ≥95%,) was used to prepare stock solutions in sterile distilled water. Piperine (≥97%,) was procured and stock solutions were prepared using Dimethyl Sulfoxide (DMSO) as the solvent, owing to its reduced solubility in water. Both Stocks were diluted as required for use and stored at 4°C. Microbroth dilution method was used to determine the MIC of the phytomolecules against Staphylococcus aureus.

The plates were inoculated with 5ul of the standard inoculum in a total volume 200ul of the phytomolecule concentrations and incubated. Inhibition was estimated based on turbidity using an ELISA Plate reader at 540 nm and later confirmed by spread plating to determine the CFU.

2.3 Determination of Synergistic Effect

To observe the combinatory effect of the Tetracycline with each of the phytomolecules a broth dilution assay was conducted. Test tubes were prepared with MIC and half MIC concentrations of Tetracycline and the therapeutic concentrations of the phytomolecules in Nutrient broth with a total volume of 200ul, along with positive and negative controls, inoculated with 5µl of the standard inoculum and incubated for 24 hours at 37°C. The following day the cultures were serially diluted, spread on Nutrient agar plates and incubated at 37°C for 24 hours. The CFU was counted and the overall CFU/ml was calculated. This was repeated for all triplicates.

2.4 Ethidium bromide Efflux Assay

The assay was performed using a modified Agar Cartwheel method (Martins et al., 2013). Test tubes with Nutrient broth were inoculated with 50 µl of bacterial inoculum at OD 0.1 at 540 nm in saline. 0.2 µg/ml Ethidium bromide was added to the tubes and incubated for 30 minutes at 25°C to allow the expression of efflux pumps and extracellular efflux of Ethidium bromide to commence. After incubation each tube received either Limonene or Piperine at their therapeutic ranges or Tetracycline at MIC (24 µg/ml) or half MIC (12 µg/ml), along with an untreated control of inoculum and ethidium bromide. The tubes were further incubated at 25°C for 30 minutes to allow the EPI's to bind and inhibit the EPs and thus inhibit the efflux of Ethidium bromide. Upon incubation the mix was swabbed onto a Nutrient agar plate using a sterile cotton swab and incubated for growth at 37°C for 24 hours. The plates were then viewed and photographed under UV fluorescence using the ChemiDoc-It 415 Imager to qualitatively analyse efflux.

2.5 *In silico* docking study

AutoDock Vina software was used for molecular docking studies. The phytocompound Piperine was used for docking and the binding affinity was checked against some efflux pumps. The binding energy scores were calculated and analyzed.

2.6 Statistical Analysis

All data is represented as its arithmetic mean ± standard deviation and represented using a Two tailed pairwise T-Test. Statistical significance was considered when $P < 0.05$. All analysis was done using GraphPad Prism Software version 8.0.

III. RESULTS AND DISCUSSION

The bacteria were found to be susceptible to all of the listed antibiotics. However, to further understand the role of efflux pumps in antibacterial activity, Tetracycline was used as the primary test antibiotic owing to its intracellular effect. The MIC for the Tetracycline was determined to be 24 µg/mL.

Both Phytomolecules were tested against the bacteria up to a concentration of 1000 µg/mL by microbroth dilution. The MIC was determined to be greater than 1000 µg/mL the highest tested concentration. However, a bell curve of therapeutic effect was observed with moderate concentrations of 400 µg/mL, 600 µg/ml and 800 µg/ml showing some reduction in viability as compared to the controls. This is owing to the eagle effect or the paradoxical zone phenomenon where some antibacterial compounds show a bell curve of inhibition as opposed to linear increase with increase in concentration (Eagle & Musselman, 1948). However lower than and higher than the therapeutic concentration showed either no difference or slightly higher viability than the controls. Possibly owing to the putative EPI's binding to high affinity binding sites on the efflux pumps at the synergistic therapeutic concentrations, while at higher concentrations occupying low affinity binding sites to accommodate excess molecules. It may also be possible that at higher than therapeutic concentrations the phytomolecules may form complexes with the antibiotic thus reducing the bioavailability of the antibiotic. Thus, the three determined therapeutic concentrations of both Phytomolecules were tested to understand their effect in potentiating Tetracycline at MIC and half MIC.

Both phytomolecules showed synergy with Tetracycline and a significant increase in overall percent inhibition was observed as compared to the Tetracycline alone. Thus, indicating while the phytomolecules do not have antibacterial activity themselves at the therapeutic concentrations the phytomolecules potentiate the activity of Tetracycline. The fold reduction in bacterial viability as compared to the tetracycline alone, suggesting synergy is listed in the Table 1.1 and 1.2.

Concentration (µg/ml)	Fold Reduction
Tet 24 + Lim 400	26.00
Tet 24 + Lim 600	5.47
Tet 24 + Lim 800	2.81
Tet 12 + Lim 400	1.62
Tet 12 + Lim 600	1.27
Tet 12 + Lim 800	1.15

Table 1.1: Fold reduction at MIC (24 µg/ml) of Tetracycline and Half MIC (12 µg/ml) of Tetracycline (Tet) in conjunction with Limonene (Lim) at the therapeutic concentrations (400 µg/ml, 600 µg/ml, 800 µg/ml)

Concentration (µg/ml)	Fold Reduction
Tet 24 + Pip 400	26.00
Tet 24 + Pip 600	4.95
Tet 24 + Pip 800	2.00
Tet 12 + Pip 400	2.14
Tet 12 + Pip 600	1.68
Tet 12 + Pip 800	1.09

Table 1.2 : Fold reduction at MIC (24 µg/ml) and half MIC (12 µg/ml) of Tetracycline in conjunction with Piperine (Pip) at the therapeutic concentrations (400 µg/ml, 600 µg/ml, 800 µg/ml)

At MIC (24 µg /ml) both phytomolecules showed the highest fold reduction in viability at 400 µg/ml, suggesting that Tetracycline exhibits the best synergy at 400 µg/ml of Limonene or Piperine with a 26-fold reduction. Comparatively, Reserpine, a known synthetic Efflux Pump inhibitor of NorA efflux pumps in *Staphylococcus aureus*, shows a 2-fold reduction in MIC (Kumar et al., 2008). This suggests that both Limonene and Piperine show a 13 times greater fold reduction than the conventional in use synthetic EPI counterpart.

A pairwise T-Test was used to interpret if a significant change in inhibition was achieved when either of the phytomolecule were used in conjunction with tetracycline as compared to Tetracycline alone. Figures 2 and 3 show the change in inhibition when the phytomolecules are used to potentiate the effect of the antibiotic at MIC and half MIC.

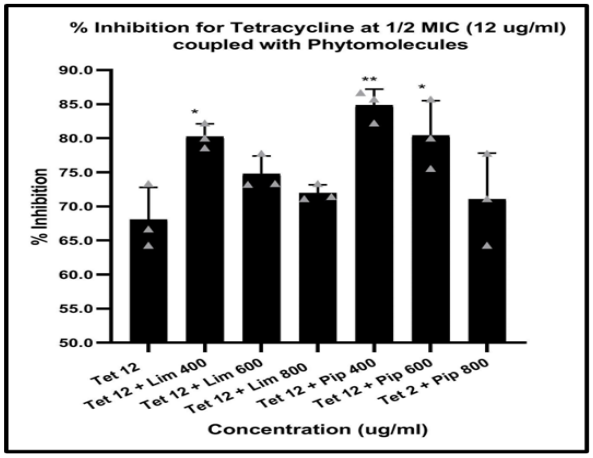

Fig 2. Percent Inhibition at half MIC (12 µg/ml) of Tetracycline (Tet 12) in conjunction with Limonene (Lim) and Piperine (Pip) at the therapeutic concentrations (400 µg/ml, 600 µg/ml, 800 µg/ml). ($P < 0.05$)

The ethidium bromide efflux assay was conducted as described and Figure 4. Depicts images of the plate's observed for EtBr fluorescence indicating efflux pump activity.

Streaks treated with EPI's are observed to have higher fluorescence owing to higher intracellular EtBr that was not efflux out owing to the presence of the EPI. The Tetracycline alone simulates the overexpression of efflux pumps by the bacterium to reduce intracellular tetracycline concentration. Owing to this Ethidium bromide is also effluxed along with the Tetracycline at a greater extent that the control (untreated) resulting in lower intracellular Ethidium bromide concentration and subsequently lower fluorescence than the control.

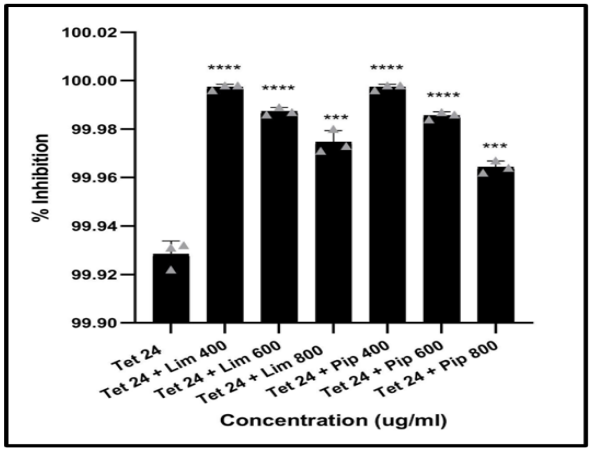

Fig 3. Percent Inhibition at MIC (24 ug/ml) of Tetracycline (Tet) in conjunction with Limonene (Lim) and Piperine (Pip) at the therapeutic concentrations (400 ug/ml, 600 ug/ml, 800 ug/ml). ($P < 0.05$)

In Img 2. significantly more fluorescence is observed in samples treated with Piperine as compared to the control. This suggests that Piperine effectively binds and inhibits efflux pumps in *Staphylococcus aureus,* preventing efflux of ethidium bromide and resulting in higher fluorescence due to higher intracellular ethidium bromide concentrations. Img 3. Shows similar results with samples treated with Limonene showing higher fluorescence than the control, further establishing limonene as an EPI for efflux pumps in *S. aureus.* This allows for the hypothesis that as the Nor family of efflux pumps are responsible for efflux of both substrates like ethidium bromide and tetracyclines (Costa et al., 2019), the increase in intracellular concentration of ethidium bromide in samples treated with the phytomolecule EPI's show that both limonene and Piperine also inhibit the efflux of Tetracycline when administered together. Img 1. Shows higher fluorescence within the control as the presence of Tetracycline in the others promotes efflux of both tetracycline and the EtBr reducing fluorescence.

Molecular Docking studies

The crystal structures of the Piperine and some efflux pumps were determined from uniport database and docked using AutoDock Vina software. Negative binding energies indicated strong binding affinity with Piperine especially efflux pumps like ABCCI and ABCGI. Interestingly, Piperine showed a higher binding affinity with norA, a known efflux pump present in *S. aureus.* Reserpine, a known EPI, also showed similar binding energy with norA.

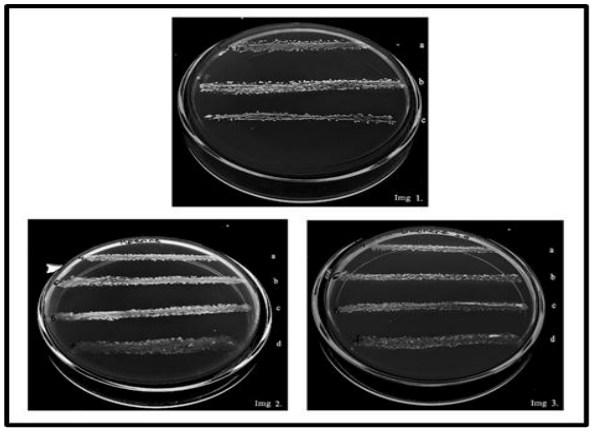

Fig 4: EtBr efflux assay

Img 1: a- Tetracycline at ½ MIC (12 µg/ml), b - Control (Untreated), c - Tetracycline at MIC (24 µg/ml).

Img 2: a - Piperine 400 µg/ml, b - Piperine 600 µg/ml, c - Piperine 800 µg/ml, d - Control (Untreated).

Img 3: a - Limonene 400 µg/ml, b - Limonene 600 µg/ml, c- Limonene 800 µg/ml, d - Control (Untreated).

Table 2: Molecular Docking Analysis

Sr. no.	Receptor	PDB ID	Score	RMSD vs Experimental Ligand
1	ABCC1	6BHU	8.229	9.761
2	ABCG2	6FEQ	6.344	18.433
3	ABCB1	8Y6H	8.805	9.430
4	SAV	2HYD (chain A)	6.312	5.745
5	SAV	2HYD (chain B)	6.354	6.057
6	NorA	5FFZ	7.710	8.542

ABCC1 (also known as MRP1 - multidrug resistance-associated protein 1, belongs to the ATP-binding

cassette (ABC) transporter group that facilitates the release of many toxic compounds and drugs out of the bacterial cell. In *S. aureus* it is associated with the development of Multiple drug resistance owing to the pump's ability to efflux such a wide variety of substrates (Costa et al., 2019 ; Van Bambeke et al., 2000). ABCG2 (also known as BCRP - Breast cancer resistance protein is another member of the large ABC transporter superfamily, ABCG2 and like the former is an efficient MDR conferring EP. ABCB1 (also known as MDR1 - multidrug resistance protein 1) is a membrane drug efflux pump that is involved in drug resistance in bacteria and other also among organisms. ABCB1 is implicated in the efflux of several antibiotics such as fluoroquinolone hence playing an important role in *S. aureus* resistance. SAV (*S. aureus* virulence factors) are the factors involved in the pathogenesis and severity of disease of *S. aureus* and include the cell surface proteins, toxins and other molecules (Costa et al., 2019). They promote immune evasion within *S. aureus*, allowing it to go undetected longer, preventing the opportunity or rapid early treatment of infection. NorA is the primary multidrug efflux pump in *S. aureus* and is commonly conserved across strains owing to its important evolutionary role in *S. aureus* survival. NorA exhibits basal constitutive expression in some strains when in a hostile environment and is essential for not only development of resistance in *S. aureus* but also secondary metabolic homeostasis (Costa et al., 2019 ; Van Bambeke et al., 2000). These tested efflux pumps and their substrates are crucial for Multidrug resistance and the first line of defense in *S. aureus* against antibiotics. The successful use of EPIs specific to these pumps is crucial in combating resistance in *S. aureus* strains by allowing for high enough intracellular levels of antibiotics to cause cell death, and on a larger scale have the potential to resolve certain MDR infections and make resistant bacteria more susceptible again.

IV. CONCLUSION

Antimicrobial Resistance poses a significant challenge to public health as the occurrence of resistance to conventional antimicrobials against previously treatable infections is on the rise. The discovery of novel therapeutics and simultaneous methods to maintain the efficacy of existing therapeutics against susceptible and resistant strains is of utmost importance. One such unexplored reservoir of therapeutics is the use of naturally occurring phytomolecules that often not only show antibacterial activity themselves but also show synergy and potentiate the activity of existing antimicrobials allowing treatments for otherwise drug-resistant infections. Exploring the functions and mechanisms of these phytomolecules might be the key to the next generation of antimicrobial therapeutics. In this paper we explored the roles of two such phytomolecules, Limonene - a monoterpene and Piperine - an alkaloid against *S. aureus* and their synergy with Tetracycline and as putative EPI's. Both phytomolecules showed a significant increase in inhibition as compared to Tetracycline alone suggesting that they not only show synergy but significantly potentiate the effect of the Tetracycline. Both phytomolecules exhibited a 13-fold greater reduction than an in-use synthetic EPI. The Ethidium bromide efflux assay allows us to further hypothesize that this synergy is owing to the phytomolecules function as a EPI in *S. aureus* possibly with some degree of specificity for the Nor family of efflux pumps, which are found in *S. aureus* and across several Gram positive bacterial species. We also prove that Piperine has affinity towards commonly expressed efflux pumps and shows a strong affinity for *Staphylococcus aureus's* primary efflux pump NorA. Thus, we conclude that both phytomolecules exhibited great potential as novel therapeutics and EPI's in combating and treating antimicrobial resistance. As part of future studies, we intend to study the nature of inhibition using fluorescence tagging of phytocompound and checking the subcellular localization inside the cell.

V.ACKNOWLEDGMENTS

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