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# *IN-SILICO* STUDIES OF THE USEFULNESS OF SOME PHYTOCONSTITUENTS AS EFFLUX PUMP INHIBITORS TO COMBAT FLUOROQUINOLONE-RESISTANT *STAPHYLOCOCCUS AUREUS*

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**ABSTRACT**

The global problem of antibiotic-resistant pathogenic microorganisms is grave. When microorganisms resist the antibiotic's effect, it is said to develop resistance. Unwanted antimicrobial exposure and irresponsible use of antibiotics have resulted in the evolution of advanced mechanisms responsible for resistance. One of such mechanism is efflux pumps, which may discharge or lower the intracellular concentration of certain antibiotics, rendering them ineffective. As a result, blocking this pathway is a feasible strategy to reduce the occurrence of bacterial resistance. This work is aimed at screening for the ability of different phytoconstituents to hinder the growth of *Staphylococcus aureus* by blocking its efflux pumps. Their effectiveness in blocking efflux pump expressed in *Staphylococcus aureus* namely NorA, Nor B, Nor C, Mep A, and Tet38 was evaluated. This was performed using Pyrx and Autodock software. As the 3D structures of NorB and Tet38 are not available, their structures were built using the I-Tasser web server. The results were evaluated with Biovia Discovery Studio. According to the results, Oleanolic acid, Ursolic acid, and Rutin had the highest binding affinity for efflux pumps of *Staphylococcus aureus*. The average binding energy of Oleanolic acid, Ursolic acid, and Rutin was found to be -9.48, -8.96, and -8.72 kcal/mol respectively. Based on ADMET analysis, Drug likeliness studies, and docking results, Oleanolic acid, Ursolic acid, or Rutin can be used as possible competitive efflux pump inhibitors and should be investigated as adjuvants in combination with antibiotics for treatment intended to reduce antibiotic resistance.

**Keywords: Antibiotic resistance, Fluoroquinolone, *Staphylococcus aureus*, efflux, Autodock software**

## 1. INTRODUCTION

The *Staphylococcus aureus (S. aureus)* is linked to various invasive disorders like endocarditis, pneumonia, and bone and joint infections. *S. aureus*-associated bacteraemia is a substantial cause of mortality both in the hospital and outside of it **[1]**. It is a highly opportunistic micro-organism that arms itself with mobile virulence encoding genetic elements and resistance determinants from other Gram-positive bacteria. Its pathogenicity is exacerbated by the fact that it has a wide spectrum of antimicrobial agent resistance mechanisms **[2]**. Antibiotic resistance occurs by modification of drug target, limiting drug uptake, drug inactivation, and drug efflux. *S.aureus* being a gram-positive microorganism without an outer membrane, drug efflux is vital which reduces the toxic compounds within the cell and hence it is a major mechanism of drug resistance **[3, 4, 5]**. Antibiotics should be able to reach their bacterial cellular targets at least at their inhibitory concentration level to show its action. This is dependent upon the rate of drug influx and efflux. *S. aureus* has been identified as having 31 efflux pumps **[6]**. The NorA efflux protein stands out among them due to its ubiquitous expression in geographically diverse strains **[7]**. and is referred to as the primary research model in the hunt for substances that impede the active efflux process **[8]**. NorA, NorB, NorC, MepA, and Tet38 are multidrug resistance (MDR) pumps that give resistance to quinolones and other agents **[9, 10]**. The largest best-characterized group of identified multidrug transporters within S. *aureus* is the Major Facilitator Superfamily (MFS). NorA is a first chromosomally identified multidrug transporter belonging to MFS and it exports an extensive range of chemicals. NorA, a hydrophobic protein has 12 putative transmembrane domains. Antimicrobial fluoroquinolone agents are widely used, because of their broad spectrum and intense bactericidal activity (Kasturi *et al.,* 2016). Fluoroquinolones are transported by the NorA efflux pump, which reduces the antibiotic's effectiveness **[11]**. Nor B is MFS proton-dependent efflux pump which influences bacterial fitness. It is constituted by 12 transmembrane segments and 463 amino acids. NorB confers resistance to some NorA substrates, including hydrophilic fluoroquinolones (norfloxacin and ciprofloxacin), biocides (tetraphenylphosphonium and cetrimide), and the dye ethidium bromide, as well as non-NorA substrates, including the hydrophobic fluoroquinolones moxifloxacin and sparfloxacin, and tetracycline **[12]**. It is noteworthy to see overexpression of NorB

during decreased aeration and acid shock. Increased resistance to NorB substrates is linked to NorB overexpression. NorC is an MFS protein with 462 amino acids and 12 transmembrane segments, and it is 61% identical to NorB. NorC is linked to low-level resistance to ciprofloxacin, moxifloxacin, garenoxacin, and other hydrophilic and hydrophobic fluoroquinolones as well as the dye rhodamine. Its overexpression results in low-level resistance since the wild-type expression of NorC is seemingly insufficient to impact the sensitivity to these chemicals. MepA is of multidrug and Toxic Compound Extrusion Family (MATE). It is encoded by the chromosomal gene mepA **[12]**. The efflux pump studies using mutants of S. *aureus* with defective NorA found MepA **[13]**. It has 451 amino acids and 12 transmembrane segments. MepA is linked to an MDR phenotype, which confers resistance to quaternary ammonium compounds. It was discovered that ciprofloxacin and norfloxacin are only marginally effective MepA substrates. Tet38 (MFS) is a membrane protein with 14 transmembrane segments **[14]**. that is involved in at least two crucial processes namely the invasion of epithelial cells by binding to the host receptor CD36 and expulsion of antibiotics and other organic entities. In recent years, the public has been interested in natural substances for treating illnesses **[15]**. A few substances that can inhibit the activity of efflux proteins have already been described in the literature; however, the clinical use of these inhibitors is hampered by poor molecular stability, poor target site selectivity, limited bioavailability, high cytotoxicity, and weak pharmacological effects **[16]**. The discovery of efflux pump inhibitors, which can restore the bacterium's sensitivity to the used antibiotic and can be combined with it to increase the effectiveness of the drug and broaden its spectrum of action, is a crucial and promising strategy to combat bacterial resistance **[17]**.

As a result of our literature review, we hypothesized that the inhibition of efflux pumps of *S. aureus* is one possible mechanism to combat antibiotic resistance using phytochemicals **[18]**. The phytochemicals like Gallic acid, Berberine, Eugenol, Resveratrol, Piperine, Quercetin, Rutin, Ellagic acid, Coumarin, Salicyclic acid, Isoflavone, Carnosol, Curcumin, Capsaicin and Olumpicin with antibiotic activity were selected. In this paper, docking-based virtual screening of these phytochemicals as efflux pump inhibitors is carried out. Also, the drug-likeliness and ADMET studies of selected phytochemicals are performed.

## 2. MATERIALS AND METHODS

### 2.1 Selection and Preparation of Receptors

The selection of receptors is done based on efflux pumps expressed for fluoroquinolones. The list of Efflux pumps and their PDB IDs is given in **Table 1 [19]**. Three-dimensional (3D) structures of NorA, NorC and MepA receptors were retrieved from the data bank of protein (https://www.rcsb.org/). The structures were visualized in BIOVIA Discovery Studio. After removal of water molecule and hetero atom from these structures, selected phytochemicals were docked using PyRx software. The 3D structures of NorB and Tet38 are not available. Hence, the I-TASSER server was employed to produce the 3D structures of NorB and Tet38. The generated models were validated using PROSA and PROCHECK web servers.

**Table 1: Selected Protein receptors of *S.aureus***

| Efflux pump | PDB ID |
|---|---|
| NorA | 1PW4 [19, 20] |
| NorB | The structure was generated using the I-TASSER model |
| NorC | 7D5P [21, 22] |
| Mep A | 3ECO [13, 23] |
| Tet38 | The structure was generated using the I-TASSER model |

**2.2 Selection and Preparation of Ligands**

Antimicrobials derived from plants have the potential to be used in the Pharmaceutical industry for the manufacturing of drugs. Exhibiting a potent antimicrobial activity, plant-based antimicrobials, either alone or combined with antibiotics, can help in dealing with the present crisis of antibiotic resistance **[24]**. Thus, herbal phytochemicals Piperine, Berberine, Rutin, Quercetin, Resveratrol, Ellagic acid, Citric acid, Gallic acid, Eugenol **[25]**, Coumarin, Salicylic acid, Theobromine, Reserpine, Oleanolic acid, Ursolic acid, Isoflavone, Carnosol, Curcumin, Capsaicin and Olympicin A **[26]**. were selected and a comparative study was performed to find the binding affinity of these phytochemical with the efflux pumps. The 3-D structures of selected ligands (SDF format) were retrieved from PubChem.

**2.3 Selection of Antibiotic**

As per the resistance data of Centre for disease dynamics Economics and Policy (CDDEP), the *S. aureus* resistance is predominantly seen in India, followed by Russia, Belarus and Turkey. *S. aureus* has majorly developed resistance to Fluoroquinolone (85%), Macrolides (78%), Oxacillin (68%) and Aminoglycoside (46%). The pace with which humans are developing antibiotics is negligible compared to the development of antibiotic resistance by microorganisms. Prudent use of the new fluoroquinolones will be required to minimize the development of resistance to these agents **[27]**.

Ciprofloxacin was selected as an antibiotic **[28]**. It inhibits the synthesis of DNA by acting on topoisomerases. The mechanisms reported for the deactivation of fluoroquinolones include synthesis of inactivating enzyme (N -acetyltransferase AAC(6′)-Ib-cr), alteration or protection of fluoroquinolone targets (DNA gyrase and topoisomerase IV; Qnr pentapeptide family proteins), alteration of Cell envelope membrane permeability (Outer membrane permeability with reduced porin production) and drug efflux pump (RND, MFS, and ABC).

**3. DOCKING PROCESS**

PyRx virtual screening software was utilized for performing molecular docking. The selected ligands were docked with the protein structures obtained from PDB or models built by I-Tasser. The binding interactions of ligands with protein were seen using BIOVIA Discovery Studio Visualizer. The binding energies of all ligands were compared. More negative binding energy value represents the most stable binding **[29, 30, 31]**.

Ligand drug-likeness parameters of three ligands were studied using the Swiss ADME and AdmetSAR - 2.0 server.

**4. RESULTS**

This study is focused on identifing appropriate adjuvants that may be used in conjunction with Ciprofloxacin to treat the antibiotic resistance of *S. aureus*. An in silico technique was utilised to evaluate the binding affinities of 20 phytochemicals with five significant efflux pumps (NorA, NorB, NorC, MepA, and Tet38) known to contribute to antibiotic resistance in *S. aureus*. The 3D structures of Nor-B and Tet38 were built using I-Tasser. Procheck analysis has given Ramachandran plot as shown in **Figure 1**. The % of amino acid in the most favoured, additionally allowed, generously allowed and disallowed regions were found to be 85, 11.7, 2 and 0.54% respectively for Tet38. For NORB, these values were 85.8, 11.7, 2.04 and 0.5% respectively. Similarly, the G-factor for both models was found to be -0.42. Ramachandran plot and ProSA z-score suggest that the homology model is properly folded and most of the protein residues are sterically appropriately placed in 3D space.

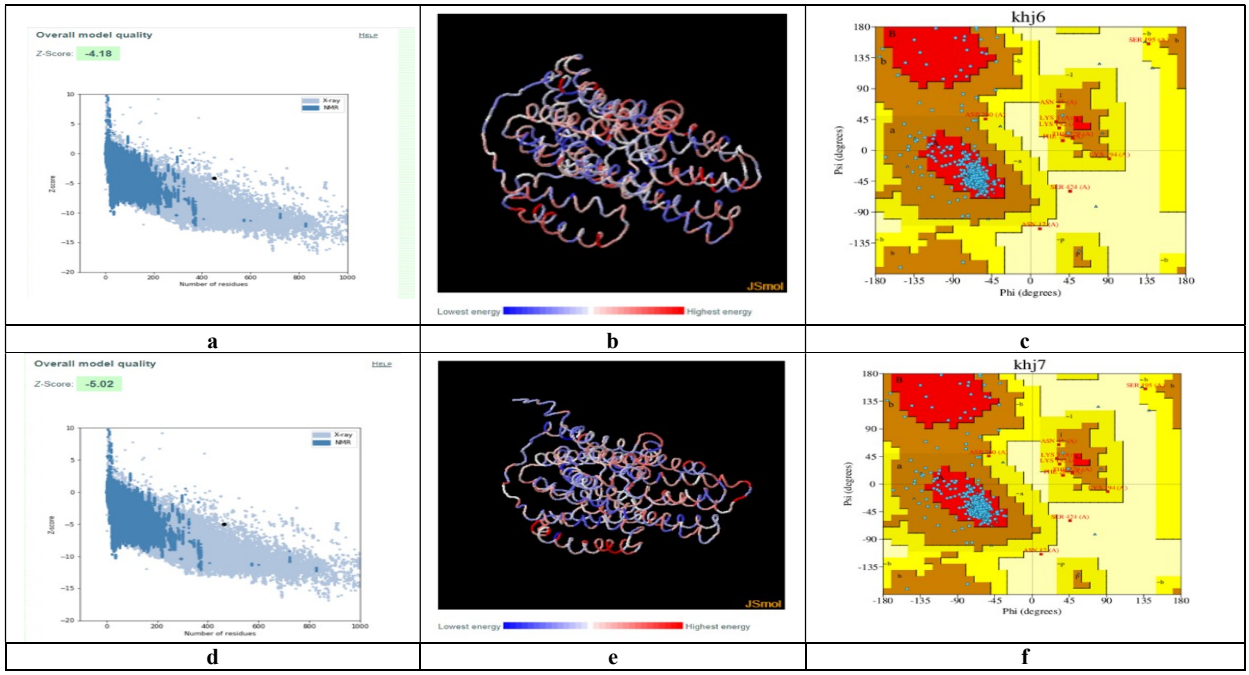


**Figure 1: a) ProSA Z score, b) Model of Tet38, c) Ramachandran plot of Tet38, d) ProSA Z score, e) Model of NorB, f) Ramachandran plot of Nor B**

The docking results demonstrated that the ligands under investigation exhibited a range of binding affinities **(Table 2)**. Three phytochemicals stood out due to their outstanding efficacy and consistently high binding affinities across all efflux pumps. The average binding energy of Oleanolic acid and Ursolic acid was found to be -9.48 and -8.96kcal/mol respectively. Similarly, Rutin has an average binding energy of -8.72 kcal/mol. These affinities raised the possibility that they may function as adjuvants in the fight against antibiotic resistance. Oleanolic acid has formed the stablest interactions with Nor B, NorC, MepA and Tet38 with the binding energy of -9.9, -8.3, -9.8 and 9.8 Kcal/mol respectively. The significant affinity that oleanolic acid has shown by interacting with each of the five efflux pumps suggests that it has a broad-spectrum inhibitory impact on these efflux pumps. Similarly, Rutin and Ursolic acid also displayed high binding affinities. These findings highlight the relevance of these phytochemicals as potential efflux pump activity inhibitors, which would boost Ciprofloxacin's efficacy. These compounds were found to bind more favourably than Ciprofloxacin. The interactions of these ligands are shown in **Figure 3, 4, 5 and 6**. Oleanolic acid has interacted with NorA with Threonine (THR A383) with a conventional hydrogen bonding formation. Van der Waals force of interaction was observed with Phenylalanine (PHE A:81), Lysine (LYS A:242), Glycine (GLY A:386), Phenylalanine (PHE A:387), Leucine (LEU A:390), Phenylalanine (PHE A:391) and Leucine (LEU A:394). With NorB, it has interacted through tryptophan

(TRP A:148) with pi-sigma bond formation, with Lysine (LYS A:402) with conventional hydrogen bonding formation and with Serine (SER A:405) with two carbon-hydrogen bond formation. Van der Waals force of interaction was observed with Tryptophan (TRP A:27, 143), Serine (SER A:147, 151), Asparagine (ASN A:280), Alanine (ALA A:283), Leucine (LEU A:286), Alanine (ALA A:378) and Tyrosine (TYR A:401). Oleanolic acid was found to bind with NorC with conventional hydrogen bonding via Glycine (GLY C:13), Arginine (ARG C:23), Serine (SER D:25). Van der Waals force of interaction was observed with Serine (SER D:11, SER C:15, SER D:15, SER D:21), Glycine (GLY C:12, GLY D:12, GLY D:13), Valine (VAL D:16), Leucine (LEU C:22, LEU D:22), and Arginine (ARG C:23). Oleanolic acid was found to bind with MepA **via** carbon-hydrogen bond with Arginine (ARG B:10), Lysine (LYS A:17). Conventional hydrogen bonds were formed with Aspartic Acid (ASP A:21) and Asparagine (ASN A:31). Van der Waals force of interaction was observed with Serine (SER B:6, SER A:13, SER B:13), Histidine (HIS B:14, HIS A:35), Methionine (MET A:16), Alanine (ALA A:20), Leucine (LEU A:24) and Phenylalanine (PHE A:108). Oleanolic acid was found to bind with TET38 via Valine (VAL A:149) having carbon-hydrogen bond formation. Van der Waals force of interaction was observed with Asparagine (ASN A:27), Lysine (LYS A:30), Leucine (LEU A:148, LEU A:295, LEU A:270), Glutamine (GLN A:269), Isoleucine (ILE A:273, 397), Glycine (GLY A:298), Tyrosine (TYR A:299), Alanine (ALA A:302, ALA A:355), and Phenylalanine (PHE A:354).

Drug likeliness parameters were generated using the Swiss ADME web server. The results are given in **Table 3**. ADMET parameters are given in **Table 4**. Compliance with Lipinski's Rule of 5 was checked to address the druggability of selected ligands. If two or more rules are violated, the usefulness of a candidate as a drug is questionable. Ursolic and oleanolic acid were found to comply with Lipinski's rule, while 3 violations were observed for Rutin. Furthermore, the toxicological features of the ligands indicated that these molecules do not exhibit Ames toxicity or carcinogenicity.

To sum up, our in silico analyses revealed that the NorA, NorB, NorC, MepA, and Tet38 efflux pumps of S. aureus displayed exceptional binding affinities for oleanolic acid, ursolic acid, and rutin. These results suggest that these phytochemicals have an array of promise for use as Ciprofloxacin's adjuvants to avoid antibiotic resistance.

**Table 2: Binding energy (Kcal/mol) of various phytoconstituents to efflux pumps**

| Sr. No. | Ligand Pubchem CID | NorA | NorB | NorC | MepA | Tet38 |
|---|---|---|---|---|---|---|
| 1. | Citric acid 311 | -5.6 | -4.9 | -5.3 | -4.5 | -5.6 |
| 2. | Coumarin 323 | -5.9 | -6.4 | -5.2 | -5.7 | -7.9 |
| 3. | Salicylic acid 338 | -5.6 | -6.1 | -5.2 | -4.8 | -6.7 |
| 4. | Gallic acid 370 | -5.7 | -5.9 | -5.3 | -5.1 | -6 |
| 5. | Berberine 2353 | -8.7 | -7.8 | -6.6 | -7.9 | -9.2 |
| 6. | Eugenol 3314 | -5.5 | -5.6 | -4.6 | -5.4 | -7 |
| 7. | Theobromine 5429 | -6 | -5.7 | -4.9 | -5.5 | -6.5 |
| 8. | Reserpine 5770 | -7.2 | -8.4 | -6.6 | -8 | -9.6 |
| 9. | Oleanolic acid 10494 | -9.6 | -9.9 | -8.3 | -9.8 | -9.8 |
| 10. | Ursolic acid 64945 | -9.6 | -9.3 | -7.7 | -8.9 | -9.3 |
| 11. | Isoflavone 72304 | -7.1 | -7.4 | -6 | -7.1 | -8.8 |
| 12. | Carnosol 442009 | -8.8 | -9.1 | -6.8 | -7.9 | -8.9 |
| 13. | Resveratrol 445154 | -7.2 | -7.2 | -5.5 | -6.4 | -8.1 |
| 14. | Piperine 638024 | -7.8 | -7.3 | -6.6 | -7.2 | -8.5 |
| 15. | Curcumin 969516 | -7.6 | -7.4 | -6.3 | -6.4 | -8.1 |
| 16. | Capsaicin 1548943 | -7.2 | -7.1 | -5.7 | -5.7 | -7.7 |
| 17. | Quercetin 5280343 | -8.2 | -8.2 | -6.4 | -7.1 | -8.9 |
| 18. | Rutin 5280805 | -9.6 | -9.1 | -7.4 | -7.9 | -9.6 |
| 19. | Ellagic acid 5281855 | -8.7 | -7.9 | -7.2 | -7.7 | -8.9 |
| 20. | Olympicin A 57380109 | 8 | -8.7 | -5.7 | -6.8 | -8.1 |
| 21. | Ciprofloxacin | -8.3 | -7.1 | -6.1 | -6.9 | -8.0 |

**Table 3: Drug likeliness parameters of selected phytochemicals**

| Sr. No. | Parameter (Ideal values) | Oleanolic acid | Ursolic acid | Rutin |
|---|---|---|---|---|
| 1. | Molecular formula | $C_{30}H_{48}O_3$ | $C_{30}H_{48}O_3$ | $C_{27}H_{30}O_{16}$ |
| 2. | Molecular weight(g/mol) (<500) | 456.70 g/mol | 456.70 g/mol | 610.52 g/mol |
| 3. | Number of H-bond acceptors (<10) | 3 | 3 | 16 |
| 4. | Number of H-bond donors (<5) | 2 | 2 | 10 |
| 5. | Log P o/w (iLOGP) (=5 or < 5) | 3.89 | 3.71 | 1.58 |
| 6. | Lipinski Violation (=1 or <1) | 1 violation: MLOGP>4.15 | 1 violation: MLOGP>4.15 | 3 violations: MW>500, NorO>10, NHorOH>5 |
| 7. | Molar Refractivity | 136.65 | 136.91 | 141.38 |
| 8. | Bioavailability score | 0.85 | 0.85 | 0.17 |
| 9. | Topological polar surface area A (=140 or <140) | 57.53 Å² | 57.53 Å² | 269.43 Å² |
| 10. | Number of rotatable bonds (=10 or <10) | 1 | 1 | 6 |

**Table 4: Toxicological characteristics of selected ligands**

| Model | Oleanolic acid | Ursolic acid | Rutin |
|---|---|---|---|
| **Absorption.** | | | |
| Aqueous solubility (LogS) | -4.38 | -4.3883 | -2.7724 |
| Caco-2 Permeability (LogPapp, cm/s) | 1.5443 | 1.5443 | -0.6508 |
| Blood-Brain Barrier | BBB+(0.7761) | BBB+(0.7761) | BBB-(0.8542) |
| Human Intestinal Absorption | HIA+(1.0000) | HIA+(1.0000) | HIA+(0.8041) |
| Caco-2 Permeability | Caco2+(0.8353) | Caco2+(0.8353) | Caco2(-0.9172) |
| P-glycoprotein Substrate | S(0.7801) | S(0.7801) | S(0.6901) |
| **Metabolism** | | | |
| P-glycoprotein Inhibitor | NI (0.8511) | NI (0.8511) | NI (0.8759) |
| CYP450 2C9 Substrate | NS (0.8258) | NS (0.8258) | NS (0.7639) |
| CYP450 2D6 Substrate | NS (0.8973) | NS(0.8973) | NS(0.8962) |
| CYP450 3A4 Substrate | S (0.7901) | S(0.7901) | NS(0.5374) |
| CYP450 1A2 Inhibitor | NI (0.9169) | NI (0.9169) | NI (0.8673) |
| CYP450 2C9 Inhibitor | NI (0.9071) | NI (0.9071) | NI (0.9071) |
| CYP450 2D6 Inhibitor | NI (0.9485) | NI (0.9485) | NI (0.9545) |
| CYP450 2C19 Inhibitor | NI (0.9025) | NI (0.9025) | NI (0.9025) |
| CYP450 3A4 Inhibitor | NI (0.8695) | NI (0.8695) | NI (0.9249) |
| CYP Inhibitory Promiscuity | Low (0.9046) | Low (0.9046) | Low (0.6787) |
| **Toxicity** | | | |
| Human Ether-a-go-go-Related Gene Inhibition | WI (0.9582) | WI (0.9582) | WI (0.9814) |
| | NI (0.8130) | NI (0.8130) | NI (0.7469) |
| AMES Toxicity | Non-AMES toxic (0.8490) | Non-AMES toxic (0.8490) | Non-AMES toxic (0.5118) |
| Carcinogens | NC (0.9394) | NC (0.9394) | NC (0.9608) |
| Acute Oral Toxicity | III (0.8316) | III (0.8316) | III (0.5971) |

Note: NS: Nonsubstrate; S: Substrate; I: Inhibitor; WI: Weak inhibitor; NI: Non-inhibitor; T: Ames toxic; NT: Non-Ames toxic; NC: Non-carcinogenic

## 5. DISCUSSION

It can be observed from the in-silico molecular docking studies that Oleanolic acid, Ursolic acid, and Rutin can effectively bind with the efflux pumps present in *S. aureus* namely NorA, NorB, NorC, MepA and Tet38. In comparison to Ciprofloxacin their co-administration with fluoroquinolone could effectively combat drug resistance in *S. aureus.* In the present study, the 3D structures of NorB and Tet38 were developed using the I-TASSER web server. They were also validated using PROSA and PROCHECK analysis. The present study also provides additional properties such as drug-likeness, and ADMET prediction essential in the development of safe and effective dosage forms. It was found that Oleanolic acid and Ursolic acid complied with Lipinski's rule. All three compounds were found to be safe for the development of formulations.

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