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Evaluation of the inhibitory effect of caffeic acid and gallic acid on *tetR* and *tetM* efflux pumps mediating tetracycline resistance in *Streptococcus* sp., using computational approach

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ABSTRACT

Background: Emergence of *tet*-efflux pump based tetracycline resistance in *Streptococcus* spp. is quite alarming worldwide posing a serious impediment in the treatment process. This leads to the search of novel target proteins to develop newer drugs against tetracycline resistant *Streptococci* spp. Caffeic acid and gallic acid being vital phenolic compounds might target the *tet* based efflux pumps. The aim of the present study is thus to explore the inhibitory potential of caffeic acid and gallic acid against *tet*-efflux pump mediated tetracycline resistant *Streptococci* spp.

Materials and methods: 3D structure of *tetR and tetM* was retrieved from the PDB data bank with further optimization of both the protein and ligands. *In-silico* inhibitory potential of the selected ligands against *tetR and tetM* was done by AutoDock 2.0 and was visualized with Accelrys Discovery Studio Visualizer tool with the assessment of the molecular properties of the ligands by molinspiration calculations and further assessment for their drug likeliness.

Results: Caffeic acid seem to possess promising inhibitory activity to target *tetR and tetM* with a promising binding energy of –5.93 and –4.6 Kcal/mol with 7 and 6 hydrogen bonds respectively. Molinspiration assessments showed zero violations with TPSA values < 140 Å towards the best oral bioavailability.

Conclusion: The findings of the study emphasize that caffeic acid and gallic acid to possess a promising inhibitory effect against *tetR and tetM* of *Streptococci* spp. suggesting caffeic acid and gallic acid as the best drug candidates to combat *tet*-pump mediated tetracycline resistance with further *in-vivo* validation targeting the same.

1. Introduction

Cell membrane bound efflux pump mediated drug resistance against single or multiple antibiotics has spurred serious concern in the treatment strategies of many systemic infections caused by pathogenic bacteria. *Streptococcus* spp. the gram positive cocci in short chains, exhibit a high level of tetracycline resistance mediated by these efflux pump mechanisms (Jin-qi Sun et al., 2017). Efflux pumps related to resistance are proteinaceous transporters that play a vital role in the extrusion of the antibiotics especially tetracylines, in the cells that harbor pBR322 plasmids (Chopra et al., 1992). *tetR* efflux pumps induce resistance in *S. mutans* via *tetA* family pumps that encompass two major groups with the first group comprising 12 TMS efflux pumps (*tetA, B, C and D*). The second group comprises plasmid mediated efflux pumps (*tetK and tetL*) among gram positive bacilli and cocci such as *Streptococcus* spp. classified under 14 TMS efflux pumps (Malhotra et al., 2005).

tetR efflux pumps reduce the accumulation of the drug inside the bacterial cells, leading to a slow phase of drug action making the cocci to adapt and acquire resistance *via* mutation or the drug targets (Rodriguez et al., 2003). In addition, the over-expression of the *tetR* efflux pumps will enhance the resistance property too (Thornton et al., 2015). Similarly, *tetM* of *Enterococcus* spp. mediated by plasmid plays a vital role in mediating tetracycline and

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tigecycline resistance (Fiedler et al., 2016). Targeting these *tetR* and *tetM* efflux pumps is one of the novel approach to curb the emergence of tetracycline resistance among *Streptococcus* spp.

Amidst various bioactive compounds, phenolic compounds from a variety of higher plants, trees and herbs are known to confer protection against the free radicals and tissue injuries (Soares, 2002). Phenolic compounds vary in its form and existence as simple phenolic and polyphenolic compounds (Burns et al., 2001). Caffeic acid, chemically known as 3,4-dihydroxycinnamic acid is one such representative of the polyphenol popularly applied in several medications such as carcinogenic inhibitor (Greenwald, 2004), antioxidant property (Gulçin 2006) antibacterial activity *in-vitro* (Dos Santos et al., 2018). It also plays a vital role in the prevention of systemic ailments such as atherosclerosis and cardiovascular diseases (Sanchez-Moreno et al., 2000). Similarly, gallic acid may occur chemically as a free from of acids, esters, catechins, tannins or as methylated forms or as catechin conjugates (Tang et al., 2003) with potent bioactivities (Borges et al., 2013).

Caffeic acid and gallic acid being considered as effective phenolic acids shows no studies conferring its inhibitory effect or their synergistic roles on the *tetR* and *tetM* efflux pumps in *Streptococcus* spp. With the analyzed reports on novel phenolic compounds, caffeic acid and gallic acid were chosen in the present investigation based on the molinspiration parameters amidst various other phenolic compounds to target the tetracycline resistant *Streptococcus* spp. The aim of the present investigation was thus to assess the *in-silico* docking analysis of the compounds with *tetR* and *tetM* efflux pumps using bio-informatics tools and to evaluate the inhibitory effect of gallic acid and caffeic acid against tetracycline resistant Streptococcus spp.

2. Methods:

2.1. Retrieval of tetR and tetM proteins and their optimization:

The crystal structure of *tetR* and *tetM* were retrieved from RCSB protein data bank (http://www.rcsb.org/pdb). Hydrogen atoms, solvation parameters and fragmental volumes to the protein were added and electronic charges were assigned to the protein atoms using kollman united atoms force field by using Auto Dock Tool (ADT) –2.0.

2.2. Ligand preparation and optimisation:

Using Chemsketch Software the structures of the caffeic acid and gallic acid were drawn together with the generation of their 3-D structures and optimization. The selected ligands were retrieved in SDB format which were further saved in.mol file followed by the subsequent conversion using open babel molecular converter program (Boyle et al., 2011) and were saved in PDB format.

2.3. Molinspiration assessment of the molecular properties of the selected compounds

The physiochemical and the pharmacological properties such as logP, hydrogen bond donor and acceptor characteristics, molecular size and rotatable bonds for caffeic acid and gallic acid were predicted by molinspiration server (Jarrahpour et al., 2012). Based on the Lipinsky's rule of five (Lipinski et al., 2001) characterization of the absorption, distribution, metabolism and elimination (ADME) of the selected compounds with further assessments and estimations of the molecular properties of the selected ligands was assessed. Membrane permeability and bio-availability was also evaluated.

2.4. Docking simulations & interpretations:

The docking analysis to interpret the affinity between caffeic acid and gallic acid against *tetR* and *tetM* of *Streptococcus* sp., was achieved by auto-dock tool with the intermediary steps such as pdb.qt files for the proteins and the ligands. Using graphical user interface program Auto-Dock tool (ADT) the grid box creation was completed with a grid size of 126x126x126 xyz points. Using Lamarckian genetic algorithm (LGA), docking simulation was achieved by setting the initial position, orientation and torsions of the ligand molecules in a random position. 10 different runs set to terminate after a maximum of 250,000 energy evaluations was used for each docking experiment with the population size set at 150. A translational step of 0.2 Å, quaternion and torsion steps of 5 were applied for each dock. The most favorable free energy of binding is achieved by clustering the results > 1.0 Å in positional root-mean-square deviation (RMSD) (Blum et al., 2008). Finally, the pose was extracted and aligned with the receptor structure with the lowest binding energy or binding affinity for final analysis.

2.5. Docking visualisation

The protein-ligand interactions like hydrogen bonding and other non-bonded energies between caffeic acid and gallic acid against *tetR* and *tetM* of *Streptococcus* spp. were visualized using Accelrys Discovery Studio Visualizer software that displays an output file and the binding area of the ligand at the surface of the protein. The relative stabilities were evaluated using their molecular dynamics, binding affinities, energy simulations with further docking score assessments.

3. Results

3.1. Structure retrieval of the tetR and tetM from Streptococcus spp.

The crystal structure of *tetR* and *tetM* from *Streptococcus mutans* (Serotype C, strain ATCC 700617) and *Enterococcus faecalis* were downloaded from PDB database. The structure ID was documented as 3VMP "A" chain and 3J25 – "A" respectively (Fig. 1). Removal of the water molecules and final stage merging of hydrogen atoms to the receptor molecule was successful. The 3D structure of *SAP-1* was visualized using RASMOL with the analysis of pink color indicating the alpha-helix, yellow arrow indicating the beta sheets and white color indicating the turns (Fig. 2).

3.2. Structure retrieval of the caffeic acid and gallic acid (the ligands):

The ligand optimization was achieved using ACD Chemsketch and retrieved in a compatible format using Open Babel molecular converter tool. The retrieved 2D and 3D structures of the ligands and its SMILES format are shown in Table 1.

3.3. Molinspiration estimation towards drug likeliness

The bioactivity scores prediction of caffeic acid and gallic acid against *tetR* and *tetM* of *Streptococcus* spp. based on the calculation towards drug likeliness is scored and tabulated in Tables 2a and 2b. Molecular properties were calculated on the based on the Lipinski's rule of five and its components. From the molinspiration results, the n-violation values of bioactive compounds caffeic acid and gallic acid are zero satisfying Lipinski's Rule of 5. TPSA was <140 Å for all the compounds thus indicating its higher absorption and promising oral bio-availability.

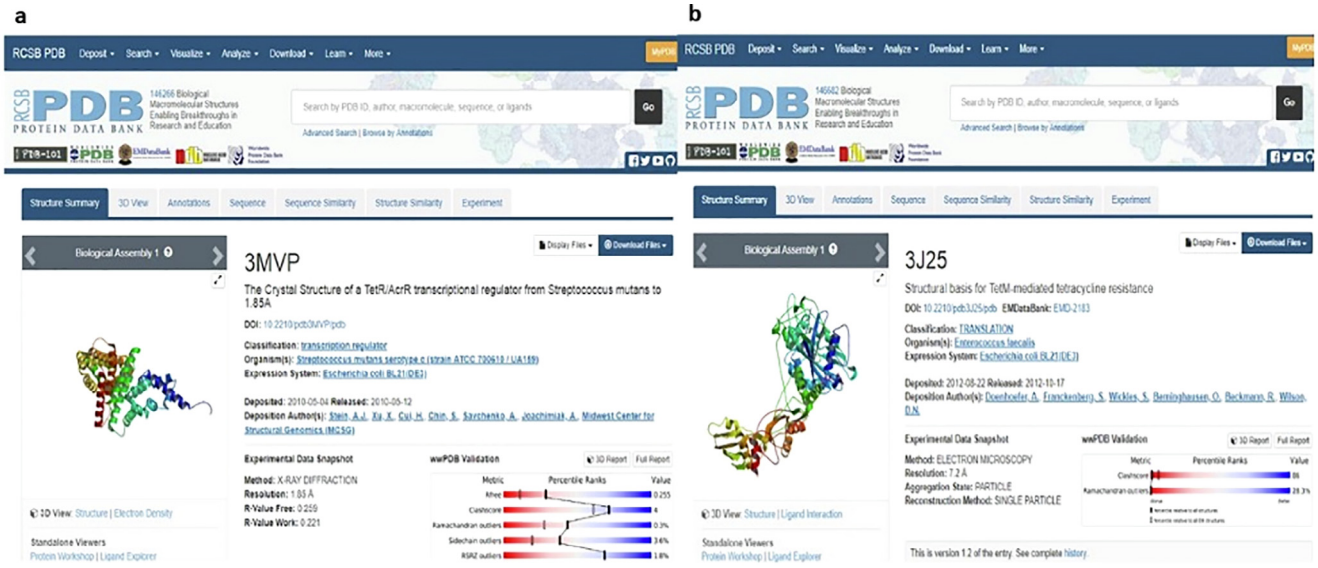


Fig. 1. Protein retrieval of Streptococcal a. *tetR* and b. *tetM* from PDB.

Fig. 2. *RASMOL 3D structure of* a. *tetR* and b. *tetM* proteins.

Table 1
2D and 3D structures and SMILES format of the selected ligands, caffeic acid and gallic acid under study.

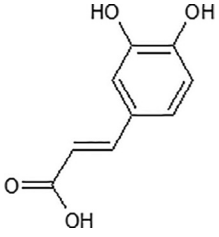
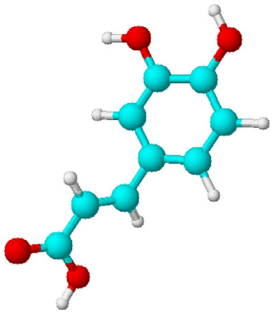
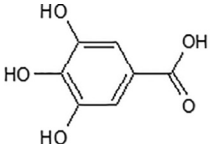
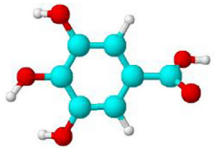
Compounds	2D	3D	SMILES
Caffeic Acid			C1 = CC(=C(C = C1/C = C/C(=O)O)O)O
Gallic acid			C1 = C(C = C(C(=C1O)O)O)C(=O)O

Table 2a
Molinspiration calculations of caffeic acid and gallic acid.

Compounds	M.wt	Mol formula	Hydrogen Bond Donor	Hydrogen Bond Acceptor	miLogP	Rotatable bonds	nViol	TPSA (Å)	Volume	N atoms
Caffeic Acid	180.15	$C_9H_8O_4$	3	4	0.94	2	0	77.75	154.50	13
Gallic acid	170.12	$C_8H_9NO_5$	4	5	0.59	1	0	97.98	135.10	12

Table 2b
Drug likeliness of caffeic acid and gallic acid.

Compounds	GPCR ligand	Ion channel modulator	Kinase inhibitor	Nuclear receptor ligand	Protease inhibitor	Enzyme inhibitor
Caffeic Acid	−0.48	−0.23	−0.81	−0.10	−0.79	−0.09
Gallic acid	−0.77	−0.26	−0.88	−0.52	−0.94	−0.17

3.4. Docking analysis of the caffeic acid and gallic acid against tetR and tetM of Streptococcus spp.

The best conformers were selected using LGA based on the best ligand-receptor structure from the docked structure based on the lowest energy and minimal solvent accessibility. Accelrys Discovery Studio Visualizer tool of the hydrogen bond interactions in stick model between the caffeic acid and gallic acid against *tetR* and *tetM* of *Streptococcus* spp. is given in Fig. 3. The amino acids of *tetR* and *tetM* binding with caffeic acid scored a promising inhibitory effect against *tetR* and *tetM* with a binding energy of −5.93 Kcal/mol and −4.6 Kcal/mol with 7 and 6 hydrogen bond interactions respectively when compared to gallic acid with, −3.92 Kcal/mol and −4.6 Kcal/mol with 4 and 5 hydrogen bond interactions. The torsional energy and the docking scores between the drug and ligands are given in Table 3. It was evident from the docking analysis that compounds caffeic acid and gallic acid possess more promising inhibitory action against *tetR* and *tetM* efflux pumps of *Streptococcus* spp.

4. Discussion

Tetracycline resistance based on *tet* gene efflux pumps, plays a vital role in varying the extent of drug resistance against cycline group of drugs especially tetracyclines in *Streptococcal* infections (Sanath Kumar et al., 2012). Amidst many mechanisms of tetracycline resistance, efflux pump based mechanisms seem to be very common. They are mostly encoded by plasmids exhibits the resis-

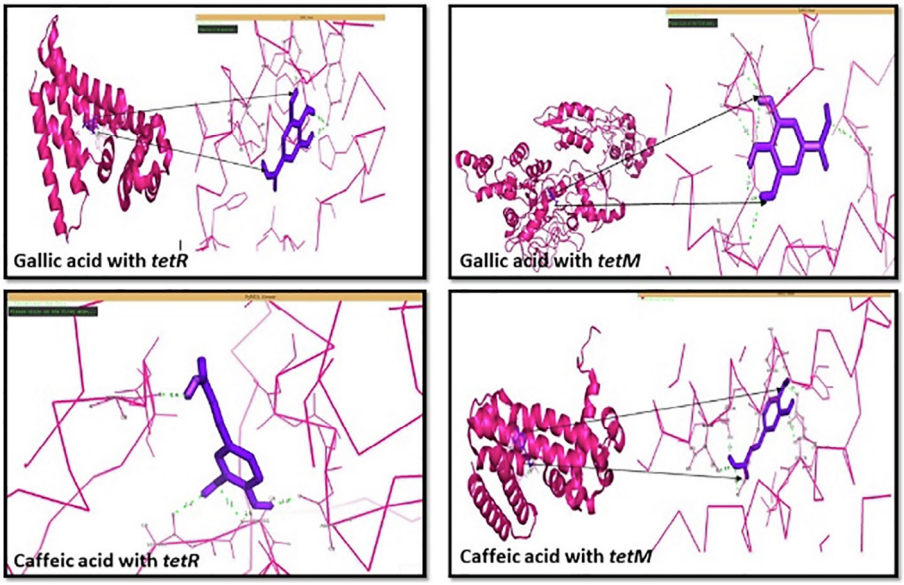


Fig. 3. Accelrys discovery studio visualisation of the hydrogen interactions between gallic and caffeic acid against *tetR* and *tetM* of *Streptococcus* sp.,

Table 3
Overall docking results of caffeic acid and gallic acid against *tetR* and *tetM* from *Streptococcus* sp.,

Molecular docking	Number of hydrogen bonds	Binding energy	Ligand efficiency	Inter molecular energy	vdW + Hbond + desolv Energy	Electrostatic energy	Torsional energy	Total internal Unbound
tetR with Gallic acid	4	−3.92	−0.33	−5.41	−5.54	−0.13	1.49	−1.18
tetR with caffeic acid	6	−5.93	−0.46	−7.42	−6.6	−0.82	1.49	−0.91
tetM with Gallic acid	5	−4.99	−0.38	−6.48	−6.13	−0.35	1.49	−0.2
tetM with caffeic acid	7	−4.6	−0.38	−6.09	−5.56	−0.53	1.49	−1.19

tance property even against higher cycline, tigecyline in *Enterococcus faecalis* (Eun-Woo et al., 2003). In the present investigation, we have made an attempt to target the *tet* based efflux pumps *viz.*, *tetR* and *tetM* from *S. mutans* and *E. feacalis*. It would be a novel method to cope the tetracycline resistance using the selected phenolic compounds caffeic acid and gallic acid which are best known for its antibacterial potential (Valéria et al., 2016). The best fit of the *tet* efflux pump proteins with the caffeic acid and gallic acid was efficiently achieved in the present investigation by molecular docking analysis which is a molecular modelling technique.

tetR and *tetM* from *S. mutans* and *E. feacalis* was retrieved from the PDB database as a desirable target based on the data recorded in database and was freely accessible. In the present study, caffeic acid and gallic acid docked against *tetR* and *tetM* resulted in a promising receptor – ligand complex. Docking analysis involves two major steps where the prediction of the exact orientation of the conformers in to the best active site pocket called pose and the strength of target-ligand binding interactions is achieved by scoring (Wang et al., 2003). Analysis using Accelrys Discovery Studio Visualizer to predict hydrogen bond interactions between caffeic acid and gallic acid and *tetR* and *tetM* yielded promising results with hydrogen bonds and bonding energies. The number of hydrogen bonds together with the enthalpic gain due to the water molecules determines the best fit (Clarke et al., 2001). In this context, caffeic acid scores to be the best inhibitory agent of *tetR* and *tetM* with a docking score of –5.93 Kcal/mol with seven hydrogen bonds respectively. This is followed by *tetM* inhibition by gallic acid with a score of –4.99 Kcal/mol again with 5 hydrogen bonds.

We used auto dock tool 4.2 which is considered as a suite of automated docking tool with a software for modelling flexible small molecule such as drug molecule binding to receptor proteins of known three dimensional structure. Using genetic algorithms for the conformational search a rapid grid based method of energy evaluation was achieved in the study in analyzing the docking simulations. In this study, the Lamarckian Genetic Algorithm (LGA) was used to explore the binding conformational landscape of caffeic acid and gallic acid docked against *Streptococcal tetR* and *tetM* efflux pump proteins. The docking scores on *tetR* and *tetM* indicated that there is a direct relationship between the energy of the binding affinity, referring to the lowest docking scores and the stability. In accordance with this, apart from the binding energy, the inter-molecular energy, vanderwaal's energy and torsional energy were also at a higher end for caffeic acid followed by gallic acid.

We performed molinspirational calculations in the present study to assess and evaluate the drug likeliness of the selected ligands. This is due to the fact that molecular properties such as membrane permeability, hydrophobicity and bioavailability are associated with some basic molecular descriptors such as log P [partition coefficient], log S [solubility], molecular weight, number of hydrogen bond acceptors and donors in a molecule are attributed to the concept of drug likeliness of the ligands and has wide acceptance in novel drug discovery and development (Leeson and Springthorpe, 2007). In the present study, molinspiration results were very promising for caffeic acid and gallic acid with the n-violations as zero satisfying the Lipinsky's rule of five suggesting the promising *tet* efflux pump inhibitory activity of the selected compounds.

In molinspiration analysis, topological polar surface area (TPSA) of a molecule is considered as a useful descriptor to characterize the drug absorption and bio-availablity and the values of TPSA and OH-NH interactions indicate that the selected ligands *viz,* caffeic acid and gallic acid to possess a smooth and efficient binding to the target proteins. However, the drug molecules with TPSA values of >140 Å or higher have low-absorption with the lipophilicity (miLogP) play their vital role in the prediction of the oral bioavailability for the drugs. In this context, caffeic acid and gallic acid score high absorption with high membrane penetration with a TPSA score of <140 Å. The scores and the binding energy between caffeic and gallic acids with the Streptococcal *tet* pumps demonstrates their potential as novel antibacterial drug candidates which could improve the treatment of intractable infections caused by tetracyclin resistant strains of *Streptococcus* spp. However, the study has its own limitations, as we did not perform the *in-vitro* inhibitory bioassays to suggest the individual bioactivity and their synergistic effect against the *tet* based efflux pumps.

5. Conclusion

Novel selection of inhibitors targeting *tetR* and *tetM* efflux pumps against tetracycline resistant *Streptococci* spp. has spurred renewed interest in recent years. The docking calculations in this study suggest the promising inhibitory effect of caffeic acid and gallic acid against *tetR* and *tetM* efflux pumps. The preliminary clue obtained from the present investigation alarms for further target based experimental screening of caffeic acid and gallic acid to combat tetracycline resistance among *Streptococcus* spp. for better selectivity with further experimental validation for the mechanism of action.

Declaration of Competing Interest

None.

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