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Identification of Biosimilar for Trimethoprim - *Andrographis paniculata* Phytochemicals Inhibits Dihydrofolate Reductase (DHFR)

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Abstract: *Staphylococcus aureus* a pathogenic bacterium responsible for hospital and community-acquired infections. Trimethoprim is generally administrated for treating *S.aureus* infection in combination with sulfamethoxazole. But increasing antimicrobial resistance towards antibiotics is a major concern. Trimethoprim targets Dihydrofolate reductase (DHFR), a crucial enzyme involved in nucleic acid and amino acid biosynthesis pathways. DHFR catalyzes the conversion of dihydrofolate to tetrahydrofolate using NADH as a cofactor. *Andrographis paniculata* is a traditionally used medicinal plant for treating various ailments, including microbial infections. More than 25 bioactive phytochemicals have been reported to exhibit various activities. The aim of the present study is to identify the lead phytochemical(s) mediating antimicrobial property of *A. paniculata* by using computational analysis. Molecular docking of *A.paniculata* phytochemicals with wild and mutated DHFR were performed. Results reveal phytochemicals interact and exhibit strong binding affinity with active site residues of wild and mutated strains. 14-deoxy-11-oxoandrographolide showed binding energy greater than 10 kCal/mol with both strains. Further analysis of *A. paniculata* phytochemicals for their efficacy would lead to the development of potential drugs for the treatment of microbial infections.

Keywords: *Andrographis paniculata*; molecular docking; *Staphylococcus aureus.*

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1. Introduction

Staphylococcus aureus, a commensal bacterium and human pathogen causes a wide range of diseases. *S. aureus* infections are mainly hospital and community-acquired. It is a major cause of bacteremia, infective endocarditis, skin and soft tissue infections, osteoarticular infections, prosthetic infections, pleuropulmonary infections, and various Staphylococcal clinical syndromes (epidural abscess, meningitis, toxic shock syndrome, urinary tract infection, and septic thrombophlebitis). *S. aureus* causes common but severe clinical infections [1-3]. *S.aureus* infections represent a curative challenge as they are associated with mortality and morbidity significantly [4]. Nearly 30% of human populations are asymptomatic nasal carriers of *S. aureus* [5]. Carriers of *S. aureus* are at elevated risk of infection and are assumed to be the main source for spreading *S. aureus* strains among individuals. Transmission of *S. aureus* occurs by direct contact, mostly skin-to-skin contact with infected individuals, and also contaminated objects and surfaces play a role [6]. Antimicrobial resistance (AMR) of bacteria from both community and nosocomial origin is emerging as a serious threat to mankind [7]. *S.aureus* categorized under priority list 2 as highly important bacteria according to World

Health Organization on the basis of drug resistance and need for antibiotics [8]. Trimethoprim (TMP) is usually administrated for treating *S. aureus* infection as Co-trimoxazole, a combination of trimethoprim and sulfamethoxazole. Increasing antimicrobial resistance of *S. aureus* is a major concern and its continuous change in the clinical disease spectrum [1].

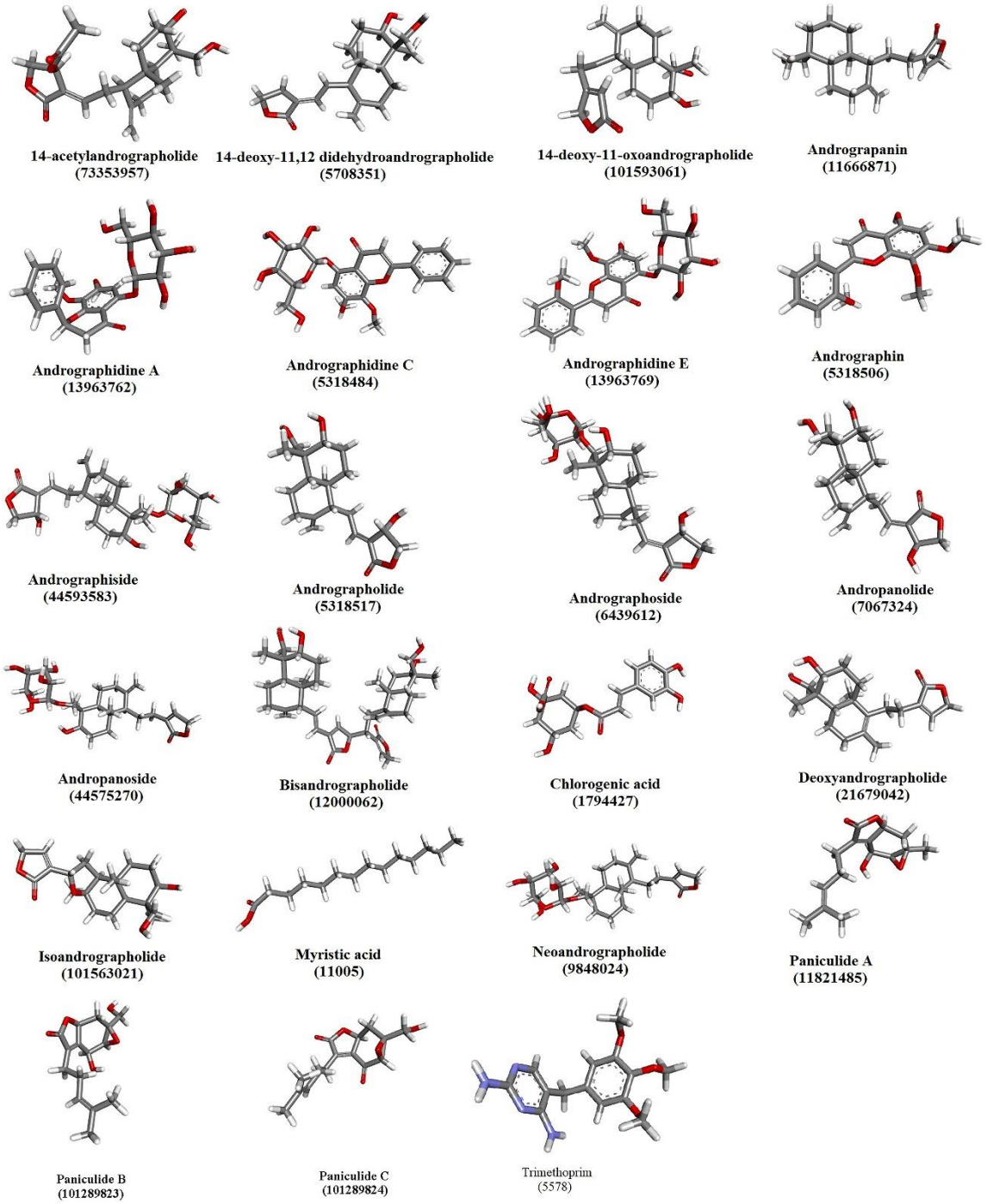

Figure 1. Three-dimensional structure of ligands.

TMP was clinically introduced in 1968, and the *S.aureus* TMP resistance was first reported in 1980s [9]. Trimethoprim resistance in *S. aureus* may raise from the chromosomal gene (dfrB) mutations or introduction of dfrA, dfrG, dfrK naturally occurring resistant genes via plasmid [10, 11]. Since the 1940s, the topical application of sulfa powder to soldier's wounds at the battlefield for inhibition of the folate biosynthetic pathway to prevent infection has been successful. Folate pathway inhibition using 'Antifolate' results in thymine-less cell death of the bacteria [12]. Dihydrofolate reductase (DHFR) is a folate-dependent enzyme

involved in various cellular components biosynthesis [13]. Hence, DHFR is considered a potential target to address the AMR towards *S.aureus* infection. *Andrographis paniculata*, an annual herb of the Acanthaceae family, is traditionally used for treating various ailments, including microbial infections. The plant has been reported for its antibacterial, antiviral, anti-inflammatory, antioxidant, and antipyretic activity [14]. Previously we have reported the presence of phytochemicals that could potentially inhibit PLAA2, a major toxic component of snake venom (15). In the present study, we propose to identify novel plant-based antifolates through computational analysis.

2. Materials and Methods

2.1. Preparation of ligands and protein.

Structures of twenty-two phytochemicals of *A. paniculata* and reference compound trimethoprim - a known inhibitor of DHFR were obtained from PubChem in SDF format (https://pubchem.ncbi.nlm.nih.gov/) and converted to PDB format using Marvin view tool. Three-dimensional structures of ligands, along with their PubChem ID are presented as Fig.1.

The PDB structure of target protein *S.aureus* Dihydrofolate reductase wild type (SaDHFR) (PDB ID: 3FRE) and mutated (SaDHFR F98Y) (PDB ID: 3FRB) were retrieved from Protein Data Bank (https://www.rcsb.org/). The three-dimensional structure of target protein *S.aureus* Dihydrofolate reductase complexed with NADPH and TMP are presented as Fig. 2.

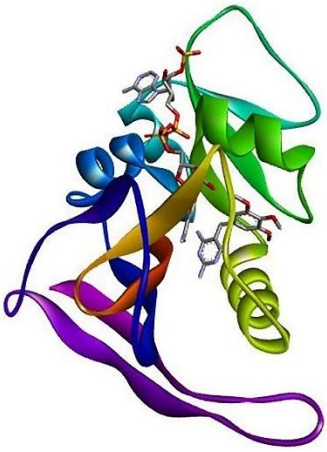

Figure 2. Three-dimensional structure of Dihydrofolate reductase complexed with NADPH and TMP.

2.2. Molecular docking analysis using Autodock.

The binding efficiency of *A. paniculata* phytochemicals with the DHFR active site was predicted using Autodock 4, an *In Silico* method. Autodock 4 uses the combination of mathematical calculations and algorithms to find the binding probability of a ligand to a protein or peptide. Autodock 4 advantage is side-chain flexibility in the protein during the ligand docking process. Autodock 4 has a free energy scoring function that works based on linear regression analysis, AMBER force field, and an even large set of varied complexes of protein-ligand with well-known inhibition constants that were used in the previous version of autodock - Autodock 3.0.

Water molecules were removed, followed by polar hydrogen bonds and Kollman charges to the target protein. Then the number of torsions was set to the ligand. Both the target protein and ligand were saved in pdbqt file format. For a ligand to bind at the target protein's

active site, a grid map was assigned with x, y, z points as 24.778, 11.673, and 38.803, respectively. Docking was performed using the Lamarckian genetic algorithm. Binding energy, binding residues, inhibition constant analyzed and produced as docking output [16].

2.3. Visualization using BIOVIA Discovery Studio Visualizer.

Docked complex of *A.paniculata* phytochemicals with target proteins SaDHFR and SaDHFR F98Y were visualized and analyzed using BIOVIA Discovery Studio Visualizer. It is a visualization and analysis suite for public use. The visualizer has many features like macromolecule design, ligand, and structure-based design and visualization. BIOVIA Discovery Studio Visualizer is an interactive three-dimensional simulation tool for visualizing and analyzing the crystal structure of small molecules, proteins, and nucleic acid.

3. Results and Discussion

Trimethoprim is a commonly used antibiotic categorized under the "Access" group of AWaRe (Access, Watch and Reserve) of antibiotics by WHO. Which is one among the top 5 antibiotics consumed worldwide [17], and also in combination with sulfamethoxazole, TMP is the 2nd top-selling FDC (Fixed-Dose Combination) in India [18]. The foremost reason behind the high consumption of TMP is its efficiency and then its least side effect. A quest on the biosimilar for trimethoprim was performed on the naturally occurring plant secondary metabolites was analyzed for potential targeting DHFR.

DHFR is a vital enzyme for all living organisms. DHFR catalyzes dihydrofolate (DHF) conversion to tetrahydrofolate (THF) using NADPH as a cofactor. Hence antifolates are one of the potential interests for addressing various diseases, especially cancer other than microbial infections. Antifolates comprise a large family of diversified compounds. Antifolates are used in treating a wide range of diseases, namely methotrexate (MTX), trimetrexate (TMTX) for cancer, trimethoprim (TMP), WR99210 for bacterial and Pyrimethamine (PYR), cycloguanil (CYC) for protozoal infection [19-23]. The folate biosynthetic pathway delivers a key strategy for designing and developing antifolates for controlling the growth of bacteria [24]. Antifolates act as a competitive inhibitor by binding at the folate-binding site of DHFR. TMP binds 2500 folds more tightly to bacterial DHFR than human DHFR [25]. Moreover, human purine synthesis depends on dietary folic acid, not endogenous folic acid; hence the host purine synthesis is not affected by trimethoprim. Antimicrobial resistance (AMR) towards trimethoprim by the *S. aureus* is due to a single amino acid substitution at the 98th position PHE to TYR, in DHFR. The mutation increased the resistance 64-fold in trimethoprim MIC [26, 27]. Hence the DHFR is identified as a potential target for antimicrobial drug development. Indigenous traditional medicinal plants are gaining importance for their remarkable efficacy in treating various diseases. Many medicinal plant extracts have been reported for their antimicrobial activity [28-32]. In the present work, the binding efficiency and interaction of various phytochemicals of *A. paniculata* with the wild type dihydrofolate reductase of SaDHFR and mutated SaDHFR F98Y of *S.aureus* was studied by molecular docking and *in silico* method using AUTODOCK 4 tool, which is largely used and has worldwide acceptance.

A.paniculata phytochemicals binding energy, inhibition constant, and interacting amino acids with SaDHFR and SaDHFR F98Y are tabulated in Table 1

Active site residues of *S. aureus* DHFR comprise LEU5, VAL6, LEU20, LEU28, VAL31, THR46, ILE-50, and LEU54 [33]. In this study, the analysis revealed the non-covalent

interactions of *A. paniculata* phytochemicals with active site amino acids of SaDHFR and SaDHFR F98Y. Bisandrographolide is the only compound that failed to show binding affinity though it interacted with active site amino acids. Two-dimensional representation of ligands (*A.paniculata* phytochemicals and trimethoprim) interaction with different targets, namely SaDHFR and SaDHFR F98Y is shown in Fig 3 and 4, respectively.

Table 1. *A. paniculata* phytochemicals and reference compound trimethoprim binding energy, inhibition constant, and interacting amino acids with SaDHFR and SaDHFR F98Y.

S.No	Ligand name	SaDHFR (PDB ID:3FRE)			SaDHFR F98Y (PDB ID: 3FRB)		
		Binding Energy (kCal/mol)	Inhibition constant	Interacting amino acids	Binding Energy (kCal/mol)	Inhibition constant	Interacting amino acids
1	14-acetylandrographolide	-9.77	68.92nM	THR121 LEU5 ASN18	-9.79	66.34nM	ALA7 ASN18 GLN95 TYR98 LEU5 LEU20 LYS45
2	14-deoxy-11,12didehydroandrographolide	-9.55	100.64nM	ALA7 ASN18 THR121 LEU20 LYS45	-9.59	92.81nM	ALA7 ASN18 LEU20 LYS45 TYR98
3	14-deoxy-11-oxoandrographolide	-10.31	27.82nM	LEU5 ALA7 PHE92 GLN95 THR121 ILE14 LEU20 ILE50	-10.11	39.0nM	ALA7 SER49 PHE92 GLN95 THR121 LEU20 ILE50
4	Andrograpanin	-9.76	70.25nM	ALA7 ASN18 ASP120 VAL6 ILE14 LEU20	-9.81	64.84nM	ASN18 ILE14 LEU20 LYS45 PHE92
5	Andrographdine A	-1.69	57.66mM	LEU5 ALA7 PHE92 ASP27 ILE50 LEU20	-1.54	74.53mM	LEU5 ALA7 ASP27 PHE92 LEU20 ILE50 GLN19
6	Andrographidine C	-6.89	8.85uM	ALA7 ASP27 PHE92 LEU28 LEU20 ILE50	-6.06	35.98µM	ALA7 ASP27 TYR98 PHE92 ILE50 ILE50 LEU20 LEU28
7	Andrographidine E	-6.22	27.5uM	ALA7 ASP27 PHE92 ILE50 LEU20 LEU28 SER49	-6.07	35.77µM	ALA7 ASP27 TYR98 GLN19 PHE92 IlE50 LEU20 LEU28
8	Andrographin	-7.2	5.29uM	ALA7 ILE14 LEU20 PHE92	-8.0	1.36µM	SER49 ALA7 ILE14 LEU20 PHE92
9	Andrographiside	-4.87	267.95uM	ASN18 SER49	-1.94	37.81mM	ASN18 ASP27

S.No	Ligand name	SaDHFR (PDB ID:3FRE)			SaDHFR F98Y (PDB ID: 3FRB)		
		Binding Energy (kCal/mol)	Inhibition constant	Interacting amino acids	Binding Energy (kCal/mol)	Inhibition constant	Interacting amino acids
				GLN95 ASP120 THR121 VAL6 ILE14 LEU20 PHE98			GLN95 ILE14 LEU20 ILE50 PHE92 TYR98
10	Andrographolide	-9.31	150.02nM	PHE202 THR121 GLY15 ILE14 LEU20 LYS45	-9.18	186.97nM	ALA7 ASN18 PHE92 VAL6 ILE14 LEU20 LYS45 TYR98
11	Andrographoside	-5.69	67.2uM	ASN18 GLN95 THR46 THR121 VAL6 ILE14 LEU20 PHE98	-3.31	3.76nM	ASP27 ASP120 ILE14 LEU20 ILE50
12	Andropanolide	-8.34	764.41nM	ASP27 THR46 SER49 VAL6 ALA7 LEU20 LEU28 VAL31 PHE92 PHE98	-9.34	142.93nM	ALA7 ASN18 PHE92 LEU20 LYS45 TYR98
13	Andropanoside	-8.34	764.41nM	ASP27 THR46 SER49 VAL6 ALA7 LEU20 LEU28 VAL31 PHE92 PHE98	-6.22	27.62µM	ASN18 SER49 LEU5 VAL6 ILE14 VAL31 PHE92 TYR98
14	Bisandrographolide	35.06	-	ASN18 TRP22 LEU5 LEU24 LEU28 PRO25 ILE50 PHE98 HIS23 VAL6 LEU20 VAL31	84.96	-	ALA7 ILE14 LEU20 LEU28 THR46 LEU54 PHE92 GLY94 LEU5 VAL31 ILE50 TYR98 PHE92
15	Chlorogenic acid	-5.89	48.52uM	LEU5 ASN18 ASP27 PHE92	-5.34	121.34µM	LEU5 ALA7 ASN18 ASP27 PHE92 TYR98
16	Deoxyandrographolide	-9.28	158.5nM	ALA7 ASN18 ILE14 LEU20	-9.31	148.89nM	ALA7 ASN18 ILE14 LEU20 LYS45 ILE50

S.No	Ligand name	SaDHFR (PDB ID:3FRE)			SaDHFR F98Y (PDB ID: 3FRB)		
		Binding Energy (kCal/mol)	Inhibition constant	Interacting amino acids	Binding Energy (kCal/mol)	Inhibition constant	Interacting amino acids
				VAL31 LYS45 ILE50 PHE92			ALA7 TYR98
17	Isoandrographolide	-9.37	135.36nM	ALA7 GLN19 THR121 LEU5 ILE14 LEU20 VAL31 PHE92	-9.38	132.55nM	ALA7 PHE92 GLN95 ASN18 ILE14 LEU20 LYS45 TYR98
18	Myristic acid	-4.86	274.31uM	LEU28 ARG57	-4.71	354.06µM	ASN18 LYS45 LEU5 ALA7 VAL31
19	Neoandrographolide	-7.78	1.97uM	ASN18 LYS45 GLN95 VAL6 ILE14 LEU20 LEU28 VAL31 PHE92 , PHE98	-6.93	8.29µM	VAL6 ILE14 LEU20 LEU28 VAL31 PHE92 TYR98
20	Paniculide A	-7.51	3.1uM	ALA7 PHE92 THR46 LEU5 ILE14 LEU20 VAL31	-7.39	3.84µM	ALA7 PHE92 LEU20 VAL31
21	Paniculide B	-7.35	4.08uM	ALA7 PHE92 LEU5 ILE14 LEU20 VAL31	-7.11	6.18µM	ALA7 PHE92 ILE14 LEU5 LEU20 VAL31
22	Paniculide C	-7.2	5.29uM	ALA7 ILE14 LEU20 PHE92	-7.25	4.84µM	ALA7 SER49 LEU5 ILE14 LEU20 VAL31 PHE92
23	Trimethoprim	-7.64	2.53uM	ALA7 ILE14 ASP27 THR121 PHE92 VAL6 LEU20	-7.96	1.48µM	ALA7 ILE14 ASP27 VAL6 LEU20 PHE92 GLY15

TYR98 of SaDHFR F98Y formed a conventional hydrogen bond with 14-acetylandrographolide, Andrographidine C and E, van der Waals interaction with 14-deoxy-11,12didehydroandrographolide, Andrographolide, Andropanolide, Chlorogenic acid, Deoxyandrographolide and pi-alkyl interaction with Andrographiside, Andropanoside, Bisandrographolide and Neoandrographolide, whereas PHE98 of SaDHFR formed an only pi-alkyl bond with Andrographiside, Andrographoside, Andropanolide, Andropanoside, bisandrographolide, and Neoandrographolide.

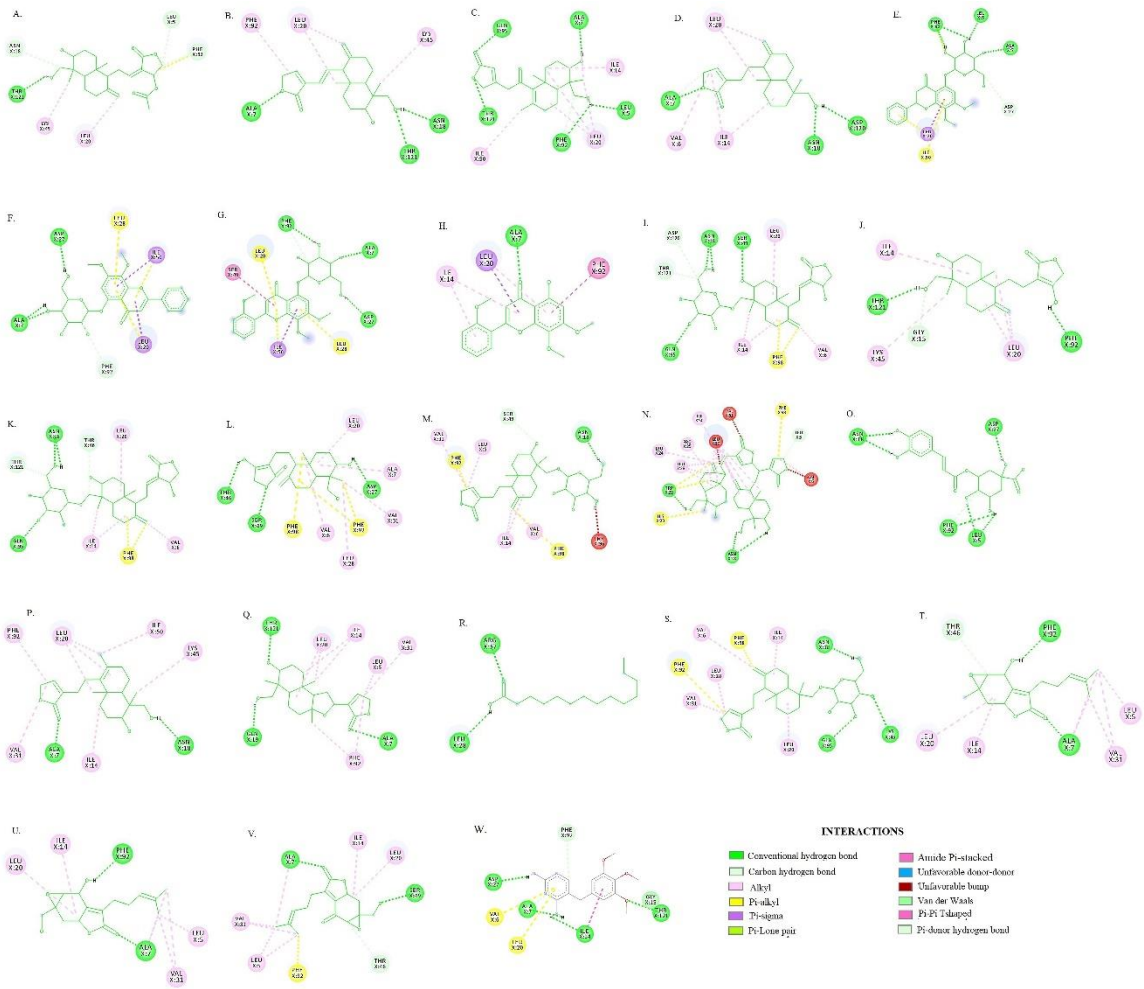

Figure 3. Two-dimensional representation of *Staphylococcus aureus* wild type Dihydrofolate reductase (SaDHFR) residues interaction with ligands. A.14-acetylandrographolide, B. 14-deoxy-11,12didehydroandrographolide, C. 14-deoxy-11-oxoamdrographolide, D. Andrograpanin, E. Andrographidine A, F. Andrographidine C, G. Andrographidine E, H. Andrographin, I. Andrographiside, J. Andrographolide, K. Andrographoside, L. Andropanolide, M. Andropanoside, N. Bisandrographolide, O. Chlorogenic acid, P. Deoxyandrographolide, Q. Isoandrographolide, R. Myristic acid, S. Neoandrographolide, T. Paniculide A, U. Paniculide B, V. Paniculide C, W. Trimethoprim.

DHFR structural analysis studies by Bhosle *et al*. 2016, GLN95 residue at supersite a druggable space in a substructure of 2W9H (Wild-type *S. aureus* DHFR in complex with trimethoprim) were unique when compared with all other clusters of supersites [34]. In our present study, phytochemicals formed a conventional hydrogen bond with the target.

14-deoxy-11-oxoandrographolide and Andrographiside formed a conventional hydrogen bond with GLN95 of both SaDHFR wild type and SaDHFR F98Y mutated whereas 14-acetylandrographolide, Isoandrographolide interacting only with SaDHFR F98Y through a conventional hydrogen bond. Andrographiside and Neoandrographolide interacted with SaDHFR.

ALA at 7th position of DHFR is shown to be a target for several phytochemicals isolated from plants and biomolecules like Chlorogenic acid, Ellagic acid, Gallic acid, Hippuric acid and Clavulanic acid [35]. Similarly, in our study, phytochemicals bind with ALA7 of SaDHFR and SaDHFR F98Y.

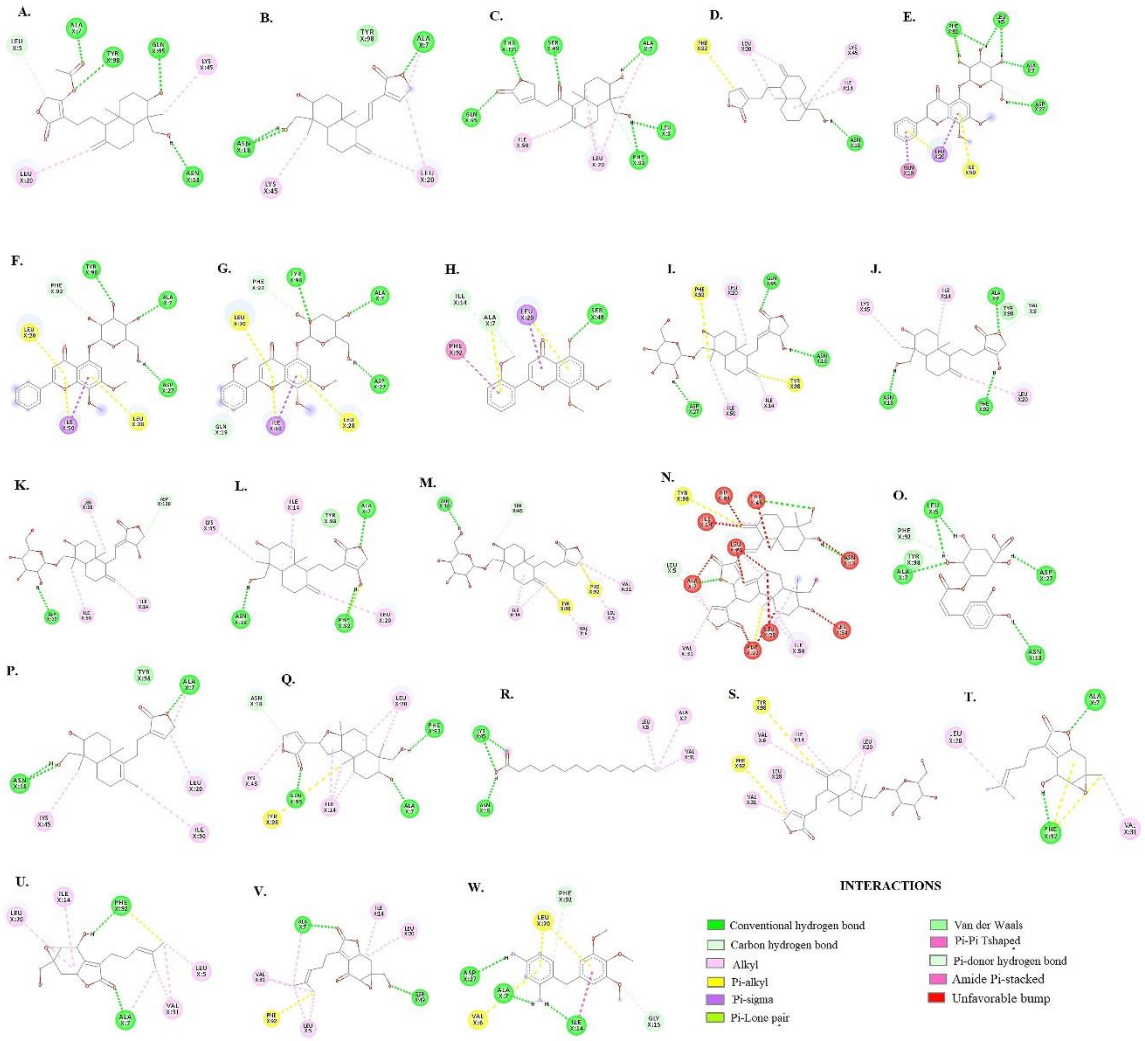

Figure 4. Two-dimensional representation of Staphylococcus aureus mutated Dihydrofolate reductase (SaDHFR F98Y) residues interaction with ligands. A.14-acetylandrographolide, B. 14-deoxy-11,12didehydroandrographolide, C. 14-deoxy-11-oxoamdrographolide, D. Andrograpanin, E. Andrographidine A, F. Andrographidine C, G. Andrographidine E, H. Andrographin, I. Andrographiside, J. Andrographolide, K. Andrographoside, L. Andropanolide, M. Andropanoside, N. Bisandrographolide, O. Chlorogenic acid, P. Deoxyandrographolide, Q. Isoandrographolide, R. Myristic acid, S. Neoandrographolide, T. Paniculide A, U. Paniculide B, V. Paniculide C, W. Trimethoprim.

The medicinal property of *A. paniculata* is well known by the scientific community and herbal medicine practitioners', of which the antimicrobial property of *A. paniculata* was reported by several scientific groups against various pathogens [36-38]. Ali *et al*. reported the inhibitory activity of *A.paniculata* methanolic extract against *S.aureus* and *E.coli* growth [39]. Mishra *et al*. 2009 reported the antibacterial activity of *A. paniculata* ethanol extract against both Gram-negative and Gram-positive bacteria [40]. Leaf extracts of *A. paniculata* showed high antimicrobial activity at 200mg/ml concentration against gram-positive bacteria *B. cereus* and *S.aureus* [41]. Antimicrobial activity of *A. paniculata* crude methanol extract and its fractions were evaluated against clinical pathogens *E. cloacae*, *E.coli*, *S.typhi*, *S.aureus*, and *C. albicans*. All the extracts exhibited antimicrobial activity against all pathogens except *E.coli* [42]. In a similar study, aqueous and different solvent leaf extracts of *A. paniculata* showed inhibitory activity against UTI (Urinary Tract Infection) bacteria [43]. Root, stem and leaves of *A. paniculata* in different solvent extracts studied for antibacterial activity against human pathogens - *Staphylococcus sp.; E.coli*, *Salmonella typhi*, *Pseudomonas sp.* Methanol extract exhibited high activity against *E.coli* followed by *S.typhi*, *Pseudomonas sp.;* and

Staphylococcus sp. [44]. Whole plant *A. paniculata* dichloromethane (non-polar solvent), methyl alcohol, and water (polar solvent) extracts were tested for antimicrobial activity by disc diffusion method at different concentrations (250, 500, 1000µg/ml) against skin disease-causing seven Gram-positive and five Gram-negative bacteria. Extracts showed substantial antimicrobial activity against both Gram-positive and Gram-negative bacteria [45]. Antibacterial activity of polar and non-polar leaf extracts of *A.paniculata* evaluated against the *S.aureus*, *S.pyrogenes*, *E.coli*, and *S.typhi*. Methanol extracts showed greater inhibitory activity than hexane extract [46]. *A.paniculata* aqueous extract, Andrographolide, the main constituent of the *A.paniculata*, and arabinogalactan protein from *A.paniculata* (dried form) exhibited antibacterial activity against *B.subtilis*, *P.aeruginosa* and *E.coli* [47]. Methanol and chloroform extracts of *A.paniculata* showed antibacterial activity against clinical pathogens [48].

Hence there exists strong evidence for the antimicrobial property of *A. paniculata*. The efficiency of phytochemicals of *A. paniculata* binding both wild type and the common mutated strain shows the possibility of solving AMR issues and problems.

The molecular mechanism of *A. paniculata* antimicrobial property and the identification of phytochemicals mediating the potent bactericidal property has not been previously reported. Hence results of the present study are the first report giving a clue on the drug target, i.e., DHFR, and the potential phytochemicals. The current result may also provoke the scientific groups to conduct further biochemical and pharmacological studies on the compounds and the whole extract of *A. paniculata* targeting DHFR, therefore addressing AMR's global issue.

4. Conclusions

AMR is a rising global issue that needs serious consideration. The probability and possibility of solving AMR with advanced technology are meager and also may require a huge budget, whereas the whole plant extracts containing numerous phytochemicals acting synergistically or non-synergistically on more than one target of infectious pathogens may address AMR shortly. Phytochemicals of *A. paniculata* have the potential to address AMR by targeting DHFR.

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Conflicts of Interest

The authors declare no conflict of interest.

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