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Repurposing drugs as uS12 ribosomal protein inhibitors to overcome UTI resistance in MDR *Pseudomonas* strains: *In silico* and *in vitro* study

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Multidrug-resistant (MDR) strains of *Pseudomonas* spp.in UTI (urinary tract infection) present a substantial worldwide health concern, requiring the development of novel approaches to identify alternative therapeutic interventions. This investigation utilizes a computational drug repurposing approach utilizing *in silico* docking studies to investigate the potential repurposing of 63 currently available non-antibiotic drugs and a control substance, streptomycin, against the uS12 ribosomal protein. The uS12 protein serves as a potential target for inhibiting translation in bacteria, potentially contributing to the mechanism by which streptomycin exerts control. The objective was to identify potential candidates that possess the capability to inhibit essential drug-resistant targets, specifically uS12. Based on our research, the observed binding energy of the control was determined to be -6 kilocalories per mole. Additionally, out of the total 63 samples tested, only 12 were found to exhibit binding energy in the range of -5.8 kilocalories per mole and above. The computational analysis of the pharmacokinetics of the 12 drugs reveals a diverse range of outcomes that support both oral and intravenous administration routes for the gathered drugs. The results of the *in vitro* minimum inhibitory concentration (MIC) analysis, Rhamnolipid, and Phycocyanin inhibition assays conducted on various strains of *Pseudomonas* spp. indicated that amlodipine, hydroxychloroquine, 5FU, Indomethacin, ascorbic acid, and calaptin exhibited higher potency compared to other drugs. The MIC values for these drugs ranged from 8.60 to 116.93 µg/mL. The results of this study show potential for expediting drug development using *in silico* repurposing methods, as well as addressing the urgent issue of antibiotic resistance in Urinary tract infections.

Keywords: *In silico* pharmacokinetics, MDR strains, Phycocyanin inhibition, Protein-drug interaction, Rhamnolipid production

Urinary tract infections (UTIs) are widespread bacterial infections affecting individuals globally, presenting a significant public health concern. The emergence of multidrug-resistant (MDR) strains of *Pseudomonas* spp., particularly *Pseudomonas aeruginosa*, has added a complex layer to the management of UTIs[1]. These MDR pathogens have evolved intricate resistance mechanisms that defy traditional antibiotics, creating a pressing need for innovative treatment strategies. MDR *Pseudomonas* spp. pose a formidable challenge in healthcare due to their exceptional resistance to multiple antibiotic classes. Their resistance mechanisms, including efflux pumps, antibiotic-modifying enzymes, and membrane alterations, render them impervious to many antimicrobial agents. The proliferation of MDR strains has led to prolonged hospital stays, increased healthcare costs, and elevated morbidity and mortality rates associated with UTIs. Conventional antibiotics, historically the cornerstone of UTI treatment, are becoming increasingly ineffective against MDR *Pseudomonas* strains, necessitating a re-evaluation of treatment approaches[2-4]. This situation has catalyzed the exploration of innovative strategies, one of which is drug repurposing – the re-evaluation of existing non-antibiotic drugs for potential utility in treating bacterial infections. Drug repurposing offers a promising avenue for addressing MDR *Pseudomonas* UTIs[5,6]. It leverages the existing library of drugs with established safety profiles, potentially expediting the availability of new treatments while minimizing the risks and costs of developing new antibiotics. This approach has particular relevance given the slow pace of antibiotic development, which can take many years and substantial resources.

Non-antibiotic drugs, initially designed for various therapeutic purposes, often possess mechanisms of action distinct from traditional antibiotics. This diversity is a critical advantage. Bacteria primarily

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develop resistance through genetic mutations or resistance gene exchange. Non-antibiotic drugs, with novel mechanisms of action, can evade these resistance mechanisms, making it challenging for bacteria to evolve resistance. Moreover, when used in combination with antibiotics, non-antibiotic drugs can enhance treatment outcomes and reduce the likelihood of resistance emergence. One of the key methodologies employed in drug repurposing is molecular docking, a computational approach that simulates the interaction between a target protein and various small molecules to predict their binding affinities[7]. Molecular docking offers the advantage of rapidly screening a diverse array of drugs, including those from different medication classes, against a specific molecular target or pathway of interest. This process allows for the identification of potential drug candidates with the capacity to modulate the target's activity, thereby serving as a foundation for further experimental validation and clinical development. Therefore, in this work, a computer-aided drug repurposing method is employed for which a variety of different antibiotic drugs and other classes of drugs have been used to evaluate the binding affinity of an uS12 ribosomal protein[8,9]. uS12 protein is a major target for translational inhibition in bacterial strains[10,11]. Streptomycin as a control drug is used in the study, and as per the finding, streptomycin binds to the uS12 ribosomal protein of the 30s subunit of the ribosome and blocks the translation process, leading to having a bactericidal effect[8,12]. Translation inhibition is one of the major pathways for bacteria killing. Finally, *in vitro* applications were analyzed of the top drugs as a result of the *in silico* studies.

In conclusion, MDR *Pseudomonas* UTIs represent a significant healthcare challenge, necessitating novel treatment approaches. Drug repurposing of non-antibiotic drugs emerges as a promising alternative, addressing the urgent need to combat antibiotic resistance, expediting treatment development, and harnessing diverse mechanisms of action. This paper explores the significance of drug repurposing, delving into its mechanisms, with a specific focus on its role in managing UTIs caused by *Pseudomonas* spp. The major objectives of the study include *in silico* docking studies involving screening the non-antibiotics drugs for the repurposing approach.ADME studies to analyze the drug's absorption, metabolism, bioavailability, distribution, and toxicity profile. *In vitro* studies include MIC50, rhamnolipid production, and phycocyanin inhibition.

Materials and Methods

In silico study: uS12 structure modeling

The 3-dimensional chemical configuration of the Small ribosomal subunit protein (uS12) was acquired using an array of computer-aided strategies. In summary, the PDB library does not include the complete isolated amino acid sequence for uS12, which is the ligand interacting domain of the drug's target, which is evident for the reference drug streptomycin. The amino acid sequence of uS12 was obtained from the UniProt database in FASTA format. The specific UniProt identifier for this sequence is Q5SHN3, and it belongs to the *Thermus thermophilus strain* ATCC 27634 / DSM 579 / HB8. The most optimal identical template was acquired using protein-protein BLAST from the National Centre for Biotechnology Information's Blast website (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to construct a three-dimensional protein structure. Using a template-based modeling approach, the Swiss Model online tool (https://swissmodel.expasy.org/) used this template for protein modeling to forecast 3D design. The structure refinement process was conducted using the Galaxy Refine online tool, accessible at https://galaxy.seoklab.org/cgi-bin/submit.cgi?type=REFINE[13]. Molecular dynamics simulation was employed as the underlying methodology.

Selection and preparation of drugs (ligands)

A total of sixty-four distinct medicines, referred to as ligands, were diligently selected to repurpose them to treat urinary tract infections (UTIs). The data used in this study were obtained from scientific papers and public databases. The chosen drugs were evaluated for conformity to Lipinski's rule of five, assessing their suitability likelihood[14]. The three-dimensional (3D) structures of the phytocompounds were obtained by downloading SDF format files from the PubChem database, accessible at https://pubchem.ncbi.nlm.nih.gov/. In addition, a conventional medicine licensed by the Food and Medicine Administration (FDA), namely streptomycin (PubChem CID: 19649), was obtained in a three-dimensional structure-data file (SDF) format and used as a control for screening objectives. The conversion of SDF data to PDB files appropriate for docking experiments was performed using the OpenBabel-2.4.1 software.

Ligand Property Prediction, ADME study, and analysis of the toxicity of the ligands

The selected ligands were assessed to determine their relationship to drug pharmacokinetics. An

essential stage in drug repurposing and drug designing involves identifying the potential for a molecule (drug) subjected to oral activity. The pharmacokinetics of drugs were assessed using Swiss ADME (http://www.swissadme.ch/index.php) to investigate their absorption, distribution, metabolism, and excretion properties. Lipinski's rule of 5 (RO5) criteria, as outlined in the field of supercomputing for bioinformatics (source: http://www.scfbio-iitd.res.in/software/drugdesign/lipinski.jsp#anchortag), were used to assess and evaluate the distinct attributes of each ligand. The desirable attributes, as per the rule, are to have a molecular weight (MW) <500 Dalton, H-bond donor <5, H-bond acceptor <10, Molar refractivity between 40-130, and logP<5 (higher lipophilicity)[14]. *In silico* potential toxicity was also evaluated by using ProTox 3.0, Tox-Prediction tool (https://tox.charite.de/protox3/index.php?site=compound_input) to access the toxicity profile of the selected drugs on various parameters such as LD50 (median lethal dose) and organ toxicity.

Molecular docking studies of drugs with a uS12 protein

The structure of the uS12 protein, generated from the Galaxy refine tool in PDB format, was examined to assess its binding energy (BE) interaction with 64 ligands. These compounds comprised 63 ligands (drugs) and one reference drug, Streptomycin. The docking parameters used in this study were selected based on established methodologies described in prior literature[15]. To facilitate docking experiments, the receptor molecule uS12 underwent preparatory steps, including the removal of H2O molecules, the addition of polar hydrogens, and the distribution of Kollman's Charge uniformly across the residues. The resulting modified structure was then stored in the PDBQT file format. Additional ligand preparation was conducted by identifying the torsion root and configuring the default rotatable bonds and torsion angles using the TORSDOF function in the Autodock program. The ligand's PDB files were converted to the PDBQT file format necessary for docking using OpenBabel-2.4.1 software. Following preparing receptor molecules and ligands, a molecular docking analysis was conducted using Autodock 4.2.6. Grid Box chose to execute blind docking to cover the whole surface of uS12 fully. Dimensions of the grid were set at 180, 135, and 150, with centers located at 0.398, 7.124, and -5.052, with a spacing of 0.375 for the x, y, and z coordinates accordingly. The Lamarckian Genetic Algorithm[16] was used to investigate the binding interaction. A five-term force field-based function called AMBER is deemed superior to other search algorithms, such as simulated algorithms or other Autodock methods. The default docking settings were used to analyze each ligand, with the exception of runs. The Lamarckian Genetic Algorithm was used in our study, with a total of 30 red for each ligand. Random starting locations and conformations were utilized, along with 2.5 million energy evaluations and an initial population size of 150. Streptomycin, an antibacterial medication often administered, was subjected to docking with uS12 in order to compare its binding efficiency (BE) with that of the selected ligands.

Determination of minimum inhibitory concentration (MIC)

The selected drugs with the highest binding affinity towards the target uS12 protein were used for the MIC analysis. MIC evaluation was a further step to narrow down the selected drugs and to obtain the most potential candidates for UTI treatment. Antimicrobial susceptibility assays were conducted to determine the minimum inhibitory concentration[17]. To brief, various bacterial colonies associated with UTI infections were isolated, selected from an agar plate, put into a Luria broth medium, and allowed to grow overnight. The bacterial suspension was adjusted to conform to McFarland's criterion of 0.5. Through dilution using a suitable medium, the cellular concentration was adjusted to 1×10^6 colony-forming units per milliliter (cfu/mL).The antibacterial efficacy of non-antibiotic drugs was assessed using the broth microdilution method. Dimethyl sulfoxide (DMSO) was employed as a solvent for drug dissolution. The chemical compound was synthesized using a stock solution with varying concentrations of 0 μg/mL, 3.12 μg/mL, 6.25 μg/mL, 12.5 μg/mL, 25 μg/mL, 50 μg/mL, 100 μg/mL, and 200 μg/mL. Streptomycin was utilized as a benchmark antibiotic for comparative analysis. A 5 μL volume of the test compound and 5 μL of cells were combined with 190 μL of LB media in a 96-well plate, resulting in a total volume of 200 μL in each well. The plate was then incubated for 24 h. Following a 24 h incubation period, a solution of resazurine dye was introduced to assess the viability of the cells. A 20 μL volume of Resazurine was introduced into each well and subjected to a 2 h incubation period. A dark purple color indicated the presence of dead cells, while a pink color indicated the presence of viable cells. The absorbance measurements were taken at wavelengths of 570 nm and 590 nm using a microplate reader. The

minimum inhibitory concentration (MIC) was determined as the lowest concentration at which no viable cells, indicated by a blue color in the resazurine assay, were observed. Cell viability was quantified and reported as a percentage.

Rhamnolipid production Assay

Rhamnolipid production was estimated by the orcinol sulfuric acid method, the detailed procedure was based on our previously reported work[18]. To brief, a standard calibration curve was made with a series of rhamnose aliquots, which were used to calculate rhamnolipid concentration. For the production analysis, different test samples were prepared based on treatment with selected drugs (amlodipine, ascorbic acid, indomethacin, hydroxychloroquine, 5FU, and calaptin), and non-treated bacterial cells were treated as control. The control sample was considered to have 100% production of bacterial strains, and accordingly, the production was compared with the treated samples. All the samples (treated and non-treated) were mixed with orcinol-sulfuric acid, incubated at 80°C for 30 min, then cooled. Subsequently, the absorbance was measured at 421 nm. The absorbance was compared to a rhamnose standard curve to determine rhamnolipid concentration.

Inhibition of Pyocyanin production

Similar to the rhamnolipid, phycocyanin (protein-pigment) is also considered an important factor contributing to the virulence of bacterial strains and quorum-sensing molecules[18,19]. Phycocyanin production was assessed following our previously reported methodology[18]. Briefly, test samples were prepared with various MIC concentrations of drugs (amlodipine, ascorbic acid, indomethacin, hydroxychloroquine, 5FU, and calaptin) and, without treated cells, were used as control. All the bacterial strain cultures were maintained, and the growth absorbance was adjusted to 0.5 at OD 600. Later, the strains were incubated with the drugs listed above at 37°C for 48 h and then centrifuged at 11,000 rpm for 10 min. Phycocyanin levels in the supernatant were measured at 691 nm. The experiment was conducted in triplicate, comparing drug effects to control strains to evaluate phycocyanin inhibition.

Results and Discussion

uS12 Protein Structure Modeling and Structure Assessment

The most effective UniProt id's sequence match for Q5SHN3 RS12_THET8. The protein data bank (PDB) identified *Thermus thermophilus* (132 amino acids) as 1VY6 with 100% sequence identity. 1VY6 template-based modeling and structure generation using ProMod3 Swiss modeling[20] yielded a 97.69% Ramachandran-favored framework. A model exhibiting GMQE and QMEANDisCo scores of 0.88 and 0.75 ± 0.07, respectively, and a QMEANDisCo>0.6 are deemed excellent standards for *in silico* study[21,22]. Galaxy Refine was employed to further enhance backbone structure quality by reorganizing side-chain atoms and relaxing the framework through molecular dynamics calculations (Fig. 1). This enhanced QMEANDisCo to 0.76 ± 0.07, and Ramachandran favored regions to 99.23% (Fig. 2), making the structure suitable for subsequent *in silico* assessment[13,23].

Drug likeliness assessment based on RO5

In the context of drug development, the Rule of 5 (RO5) predicts that poor absorption, low permeability, and poor oral availability are more likely to occur if there are more than 10 H-bond acceptors, 5 H-bond donors, a molecular weight (MW) of more than 500 g/mol, and a calculated Log P>5[14]. In other words, these factors are more likely to be limiting factors. From Table 1, it is clearly evident that Atorvastatin, Daunorubicin, Doxorubicin, and Rafoxanide, together with the standard control drug streptomycin, have a MW>500. This implies that these compounds may have problems associated with *in vivo* oral bioavailability. However, this criterion can be bypassed as the drug can be administered by I.V. (Intravenous). Likewise, other properties (H-bond donor and acceptor) suggested that Benzamil, Phenamil, Daunorubicin, Doxorubicin, Methotrexate, and streptomycin have higher H-bond donor groups. In addition to this, the last four above-mentioned

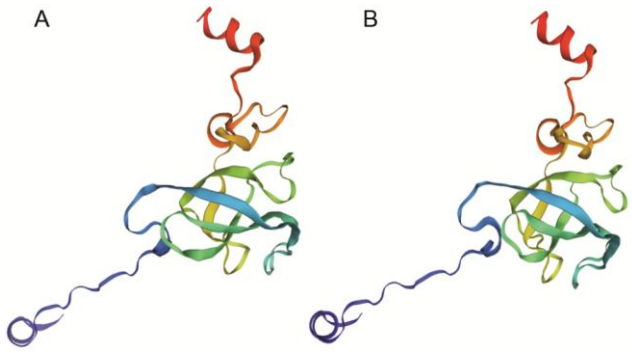

Fig. 1 — 3D PDB structure: (A) Template-based modeled structure of uS12 ribosomal protein; and (B) Refined structure of Swiss-modeled uS12 by GalaxyRefine web tool

Table 1 — Lipinski Rule of 5 for different ligands

Ligands	Molecular Mass (g/mol)	H bond donor	H bond acceptor	LogP	Molar Refractivity
5FU	130	2	4	-0.3633	25.865
Acetaminophen	151	2	3	1.3506	42.410496
Alogliptin	339	2	7	1.21678	91.731
Amlodipine	408.5	3	7	2.266	105.577065
Aspirin	180	1	4	1.31	44.71
Atorvastatin	527	4	6	2.65238	130.706558
Azathioprin	277	1	6	1.1458	65.669098
Benzamil	319.5	7	8	0.5391	85.101
Bithinol	356	2	2	5.862599	80.376
Calaptin	454	0	6	5.09308	131.655
catecholamine	125	3	3	-0.1055	32.834194
celecoxib	381	2	4	4.785319	90.319397
chlorpromazine	318.5	0	2	4.8944	92.193977
clotrimazole	344.5	0	1	5.3767	101.842972
colchicine	399	1	7	2.5861	108.182671
coumarine	146	0	2	1.6188	41.110996
cyclophosphamide	261	1	4	1.884	59.191189
daunorubicin	527	6	11	0.5635	128.476547
Diclofenac	296	2	3	4.364099	77.526
Diflunisal	250	2	3	3.035599	60.418098
Doxorubicin	543	7	12	-0.4641	129.888351
Escitalopram	324	0	3	3.812979	90.913971
Floxuridine	246	3	7	-1.1827	50.939297
Gemcitabine	263	4	7	-0.9937	54.697998
GTS21	308	0	4	3.765299	92.39798
Hydroxychloroquine	335	2	4	3.782999	98.271469
Ibuprofen	206	1	2	3.0732	61.034786
Indomethacin	357.5	1	4	3.927319	95.747276
Linagliptin	472	2	9	2.29772	133.046906
Liraglutide	464	1	6	3.992479	112.591179
Mebendazole	295	2	5	2.972199	81.995872
Meloxicam	351	2	7	3.03172	86.464493
Metamizole sodium	311	1	7	1.9694	78.613785
Metformin	129	5	5	-1.24383	37.223495
Methotrexate	454	7	13	0.2684	118.261597
Mirabegron	396	5	6	2.7722	113.27758
Mitomycin	334	5	9	-1.6512	80.110489
Mitotane	320	0	0	5.929001	80.24099
Niclosamide	327	2	5	3.859499	79.176384
Oxaprozin	293	1	4	3.645779	82.009773
Oxyclozanide	401.5	3	4	5.617099	89.216774
Parthenolide	248	0	3	2.762	68.012985
Penicillamine	149	3	3	0.1067	38.676193
Phenamil	305.5	7	8	0.6706	81.057388
Promethazine	284	0	2	4.239399	87.16198
Rafoxanide	628	2	4	6.952801	124.47197
Raloxifene	473	2	5	6.075202	136.251099
Salbutamol	239	4	4	1.306	66.745079
Saxagliptin	315	3	5	1.15798	82.799179
Simavastatin	418	1	5	4.585599	115.45475
Sitagliptin	407	2	5	2.0165	83.053398

(*Contd*.)

Table 1 — Lipinski Rule of 5 for different ligands (*Contd*.)

Ligands	Molecular Mass (g/mol)	H bond donor	H bond acceptor	LogP	Molar Refractivity
Tamoxifen	371	0	2	5.996099	119.581955
Thioridazine	370	0	2	5.885601	110.67997
Topiramate	339	2	9	0.685401	71.648384
Topotecan	421	2	8	1.4605	111.618561
Toremifene	405	0	2	6.215	124.627953
Tretinoin	300	1	2	5.6026	93.76178
Verapamil	454	0	6	5.09308	131.655975
Vildagliptin	303	2	5	1.17428	80.713478
VitamineB1	265	3	4	0.60774	70.324188
VitamineB6	169	3	4	0.08022	42.484394
VitamineC	176	4	6	-1.4074	35.256191
VitamineE	430	1	2	8.840264	134.390778
Streptomycin (Control)	581.6	12	15	-8.1611	132.918884

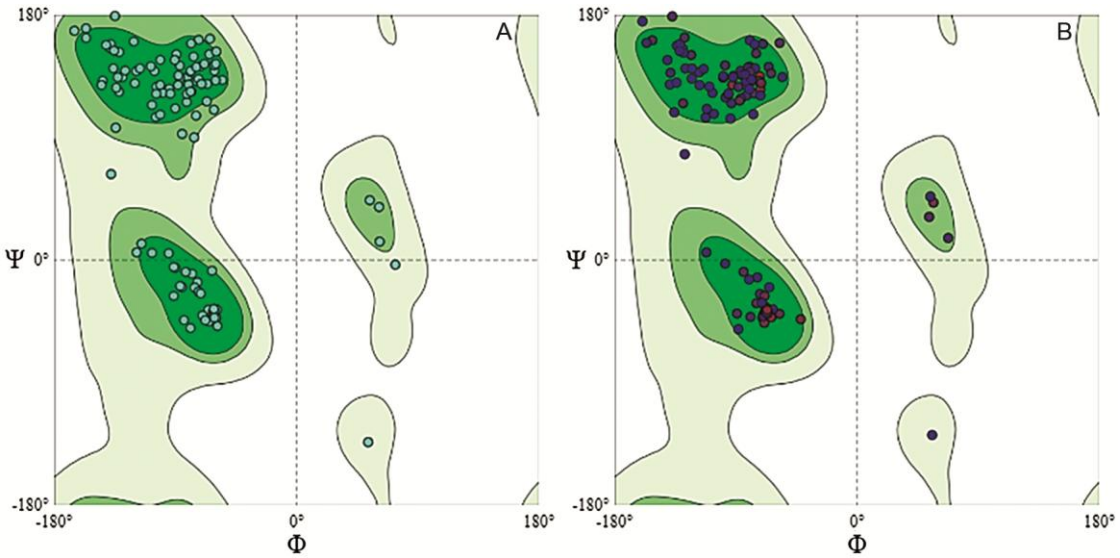

Fig. 2 — Ramachandran plot: Showing dark green color region- most favorable, light green color region- allowed conformations, the lightest green region as fairly allowed region, and white color represents- disallowed regions. (A) Swiss modeled structure showing a rama-favored region of 97.69%; and (B) Refined model structure showing a rama-favored region of 99.23%

compounds have H-bond acceptor groups above 10. Molar refractivity observations don't show a much greater variation from the desired values i.e., between 40-130. In contrast to this, logP values of Rafoxanide, Mitotane, Bithinol, Raloxifene, Tamoxifen, Thioridazine, and Vitamin E show a greater variation while the remaining drugs are within the limit of desired logP value <5. All the RO5 calculations suggest that the above-mentioned drugs are not suitable candidates for oral bioavailability and delivery. In this regard, several studies have quoted that strict following of RO5 may not be 100% validated as some FDA-approved drugs don't obey RO5 but are used as drugs. Such examples are atorvastatin, digitoxin, erythromycin, and montelukast, all of which have MW>500 daltons[24]. Studies also suggest that drugs of natural origin are orally active and do not follow all the parameters of Lipinski's rule[24]. As a result, one may draw the conclusion that the parameters for orally active drugs provide a delicate balance between the odds of discovering a compound with drug-like properties and the elimination of potential candidates for further biological testing. On another note, IV medicines are more bioavailable than oral ones. IV drugs are rapidly absorbed, increasing bioavailability[25]. Therefore, all the compounds were selected for *in silico* molecular docking studies to screen out the best matches for the drug repurposing for UTI treatment.

Docking studies: Ligplot analysis for Active (Binding) site

The Binding Energy (BE) is an evaluating factor of the strong bonding interaction between receptors and ligands. The more negative the BE is, the more the affinity of the drug (ligand) with the target[26]. 64 bioactive drugs (63 drugs + Streptomycin reference) were tested against uS12 ribosomal protein for this molecular docking study. The number of participating amino acids in H-bonding, RMSD (Root Mean Square deviation: the average deviation between the corresponding atoms of proteins-ligand interaction), and BE (kcal/mol) were recorded in (Table 2). All the Protein-drug interactions were performed in the designated grid boxes mentioned earlier. As per the data obtained in Table 2, Streptomycin, which was used as a standard reference for the study, showed BE -6 kcal/mol and RMSD of *10.837 Å*.To process the screening selection, two criteria were implemented as per the streptomycin results: the first criterion is to have a BE equivalent or higher than 6kcal/mol, and the second criterion of have an RMSD value of less than 10.837 *Å, as smaller the value of RMSD more stable the formed structure in a dynamic simulation state*[27].As per Table 2, out of the other 63 compounds, only 13 ligands BE were similar in the range of -5.8 and above. This includes Amlodipine, Benzamil, Hydroxychloroquine, 5FU, Indomethacin, Ascorbic acid, Calaptin, Diclofenac, Paracetamol (acetaminophen), Pyridoxine, Celecoxib, Atorvastatin, and Promethazine. Whereas, on screening on the basis of RMSD, 28 drugs were shortlisted. When overlapping both the screening criteria, a common 12 drugs were selected having both the parameters as desired. Table 3 highlights the top 12 selected compounds in addition to the reference streptomycin, displaying conventional and non-conventional H-bonding, alkyl interaction details. The list encloses the amino acids involved in interactions of the individual drugs with the target uS12 protein.

Table 2 — Molecular Docking: Binding energy calculations (ΔG) for different ligands with uS12 ribosomal protein

Ligand/Drug	Energy (kcal/mol)	RMSD
5FU	-6.2	3.145
Acetaminophen	-5.9	1.462
Alogliptin	-5.5	19.983
Amlodipine	-6.1	6.638
Aspirin	-5.1	3.224
Atorvastatin	-6.2	9.63
Azathioprin	-4.5	17.842
Benzamil	-5.8	17.337

(*Contd*.)

Table 2 — Molecular Docking: Binding energy calculations (ΔG) for different ligands with uS12 ribosomal protein

(*Contd*.)

Ligand/Drug	Energy (kcal/mol)	RMSD
Bithinol	-4.7	20.254
Calaptin	-5.8	2.185
catecholamine	-3.8	17.657
celecoxib	-6.5	8.952
chlorpromazine	-4.3	20.572
clotrimazole	-5.2	20.726
colchicine	-5.1	19.612
coumarine	-4.3	19.147
cyclophosphamide	-4	3.246
daunorubicin	-5.7	19.109
Diclofenac	-6.2	2.129
Diflunisal	-5.7	10.44
Doxorubicin	-5.1	8.543
Escitalopram	-5.3	17.87
Floxuridine	-4.7	1.934
Gemcitabine	-5.2	4.547
GTS21	-4.8	18.459
Hydroxychloroquine	-6.1	5.235
Ibuprofen	-4.9	18.59
Indomethacin	-6.1	4.227
Linagliptin	-5.4	21.117
Liraglutide	-5.2	19.342
Mebendazole	-5.6	30.51
Meloxicam	-4.7	33.166
Metamizole sodium	-5.4	2.358
Metformin	-3.8	17.697
Methotrexate	-5.3	9.416
Mirabegron	-4.4	15.111
Mitomycin	-5.3	18.886
Mitotane	-4.9	18.523
Niclosamide	-4.6	9.013
Oxaprozin	-5.6	14.018
Oxyclozanide	-5.5	9.912
Parthenolide	-5.3	20.423
Penicillamine	-3.8	14.107
Phenamil	-5.6	18.152
Promethazine	-5.9	4.642
Rafoxanide	-4.3	2.362
Raloxifene	-3.9	3.524
Salbutamol	-4.3	17.315
Saxagliptin	-5.6	3.129
Simavastatin	-5.6	14.351
Sitagliptin	-5.4	24.135
Tamoxifen	-5.3	19.806
Thioridazine	-4.9	21.011
Topiramate	-4.9	19.658
Topotecan	-5.2	8.75
Toremifene	-5.1	19.844
Tretinoin	-5.3	22.097
Verapamil	-3.3	22.684
Vildagliptin	-4.7	2.588

(*Contd*.)

Table 2 — Molecular Docking: Binding energy calculations (ΔG) for different ligands with uS12 ribosomal protein (*Contd*.)

Ligand/Drug	Energy (kcal/mol)	RMSD
VitamineB1	-3.8	4.396
VitamineB6	-6.1	8.592
VitamineC	-5.9	2.022
VitamineE	-5.6	22.159
Streptomycin (Control)	-6	10.837

The top 12 selected compounds, as per Table 3, and their individual ligand-uS12 protein complex wereanalyzed in the discovery studio visualizer and pymol to understand bonding patterns and their 3D and 2D interactions. Finally, the active site bonding pattern was drawn on Ligplot+ v2.2, and (Fig. 3) depicts the possible interaction between uS12 with selected drugs and streptomycin. As per Figure 3, the involvement of mainly Arginine, Lysine, and Glycine

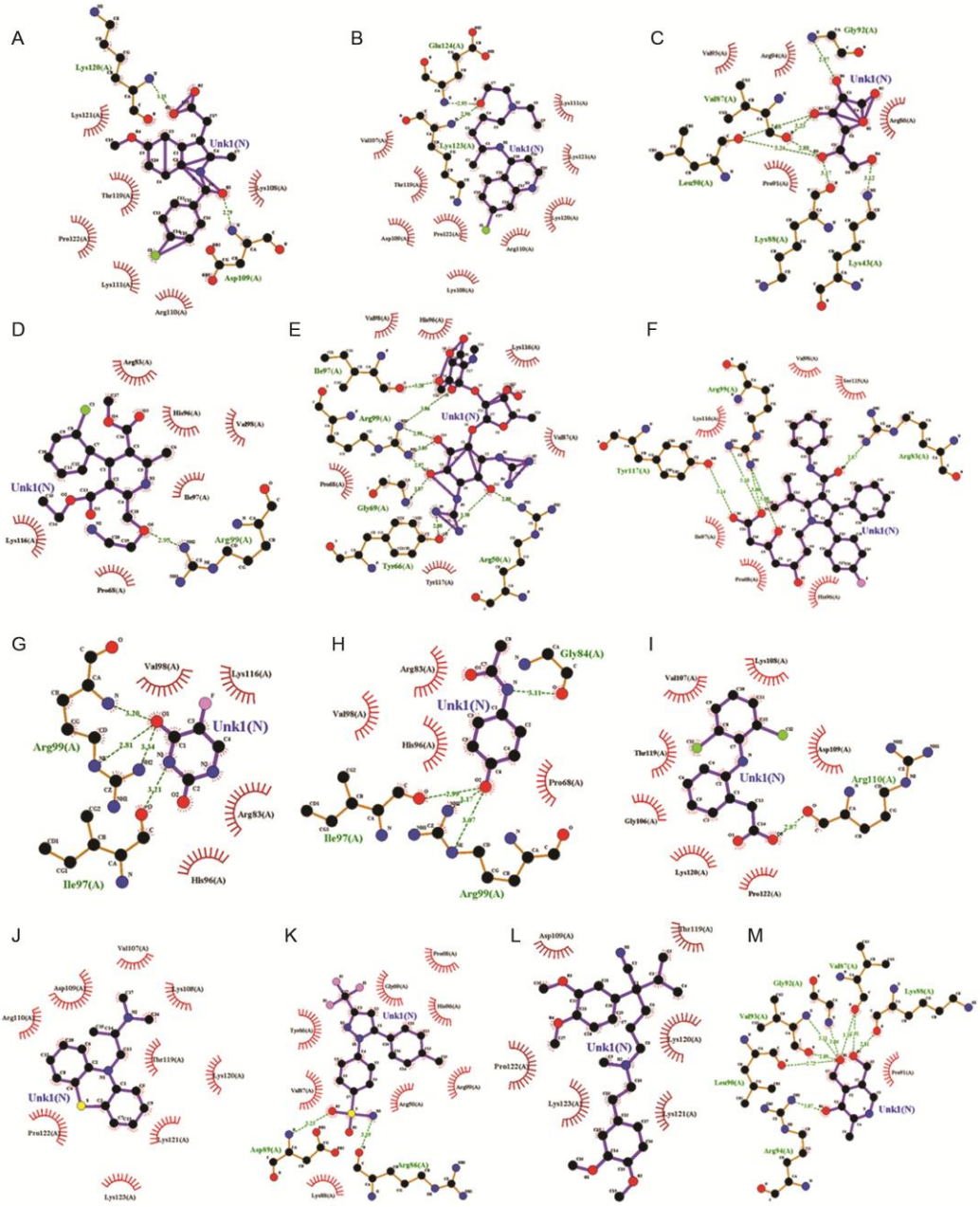

Fig. 3 — Ligplot chart showing the amino acid interaction of uS12 protein with different drugs, where values written in green confirm the distance of the H-bonds between two atoms (A) Indomethacin; (B) Hydroxychloroquine; (C) Ascorbic acid; (d) Amlodipine; (E) Streptomycin; (F) Atorvastatin; (G) 5FU; (H) Paracetamol (acetaminophen); (I) Diclofenac; (J) Promethazine; (K) Celecoxib; (L) Calaptin; and (M) Pyridoxine

Table 3 — Binding Interaction of uS12 protein with the top 12 selected drugs

Drugs/Ligands docked with uS12	Conventional H-Bonding	Non-conventional H-Bonding	Alkyl (Pi) bonding
Amlodipine	Arg99	Arg83	Lys116, Val98
Hydroxychloroquine	Lys123, Lys120		Lys111, Pro122*
5FU	Arg99*, Ile97		Arg83
Indomethacin	Asp109, Lys120		Pro122*, Lys111, Lys120*, Lys123
Ascorbic acid	Lys43, Leu90*, Val87*, Lys88, Gly92	Arg86, Val93	
Calaptin			Lys111, Pro122*, Lys120, Lys123*
Diclofenac	Arg110		Lys120, Lys108
Paracetamol (acetaminophen)	Arg99*, Ile97, Gly84		Val98, Arg83
Pyridoxine	Val87*, Leu90, Val93*, Lys88, Gly92, Arg94		
Celecoxib	Asp89, Arg86	Tyr66	His96, Val87, Pro68
Atorvastatin	Arg83, Tyr117, Arg99*	Lys116	Val98, Pro68
Promethazine		Val107	Lys123, Pro122*
Streptomycin	Arg99*, Gly69, Tyr66, Arg50, Ile97	Val98, Tyr66	

*Represents interaction (H-bonding/alkyl bonding) of 2 or more than 2 from a single amino acid residue

amino acids in the formation of conventional H-bonds can be seen by comparing the interactions of all compounds. Whereas, Tyrosine and Valine are involved in non-conventional carbon H-bonding. In addition, Lysine, Proline, and Valine can be seen at sites having alkyl pi bonding and alkyl bonding. The specific amino acids participating in the uS12-drug interaction are listed in (Table 3).

In silico pharmacokinetics: Swiss ADME study

The selected drugs were analyzed for the *in silico* pharmacokinetics study to analyze the interaction pattern of the molecule once administered orally. We analyzed the *in silico* evaluations of the four stages of pharmacokinetics, namely absorption, distribution, metabolism, and excretion (ADME). SwissADME, a web tool that examines and determines the pharmacokinetics and drug-likeness of compounds as being based on multiple algorithms, is implemented to review the ligands for ADME attributes. ESOL value (estimated solubility), which is a method of decoding the solubility of a compound in aqueous solutions[28].As per ESOL values, the drugs can be arranged in the decreasing order of their solubility as Ascorbic acid > 5FU > Paracetamol = Pyridoxine > Streptomycin > Amlodipine > Hydroxychloroquine >Calaptin> Celecoxib > Diclofenac > Indomethacin > Promethazine > Atorvastatin. This solubility range is quite evident in the radar image as well as in (Fig. 4). GIA (Gastrointestinal absorption) and P-gp substrate are the indications of the drug absorption level[29], which predicts that almost all the mentioned compounds have high levels of intestinal absorption except atorvastatin and streptomycin, which have low levels. In relation, only amlodipine, calaptin, atorvastatin, and streptomycin are the substrates for the p-gp efflux channel, implying less drug accumulation of these drugs with respect to others that are not the substrate[30].

The distribution of the drugs within the human body was documented based on their ability to traverse the Blood-Brain Barrier (BBB), revealing that only hydroxychloroquine, Indomethacin, calaptin, diclofenac, paracetamol, and promethazine, drugs exhibited BBB permeability. Chemical metabolism predominantly takes place in the liver organ, where specific enzymes are present. Cytochrome P450 plays a pivotal role in the metabolism of toxins, drugs, and chemicals[29,30]. CYP3A4 and CYP1A2 are integral constituents of the hepatic metabolic pathway. Compounds of xenobiotic nature that exhibit inhibitory effects on these proteins lead to the accumulation of drugs and the manifestation of drug-drug interactions, resulting in toxicity[29,31]. On the contrary, the inhibition of CYP3A4 may offer benefits in alternative scenarios as it has the potential to elevate the levels of quickly metabolized drugs in the bloodstream, thereby increasing their therapeutic efficacy[31]. Based on the ADME analysis, it is observed that among the compounds studied, only Amlodipine exhibits inhibitory activity towards both

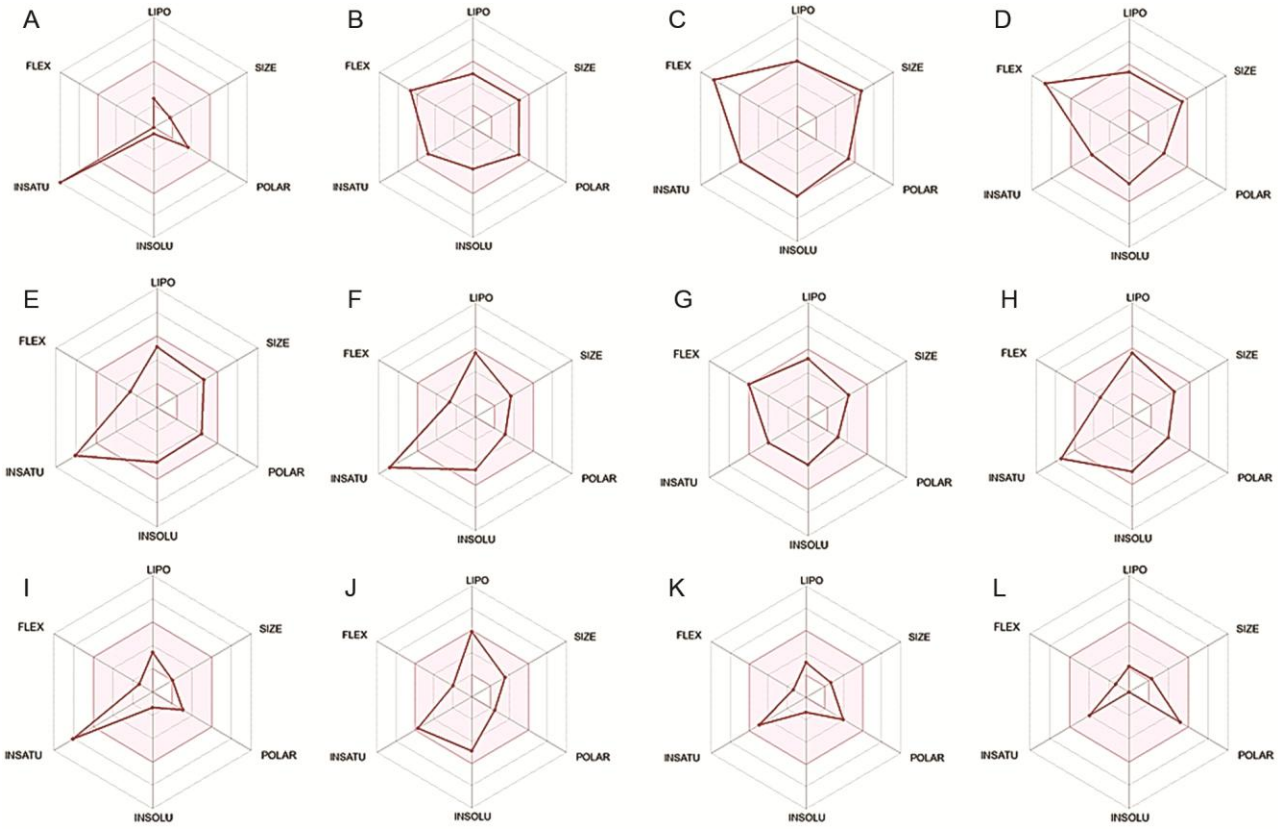

Fig. 4 — Radar images of the screened 12 drugs (*in silico* pharmacokinetics) displaying: (a) 5FU; (B) Amlodipine; (C) Atorvastatin; (D) Calaptine; (f) Celecoxib; (G) Diclofenac; (H) Hydroxychloroquine, (I) Indomethacin; (J) Paracetamol (Acetaminophen); (K) Promethazine; and (L) Pyridoxine Ascorbic acid (Vitamin C)

CYP3A4 and CYP1A2 enzymes. In contrast, the other compounds, namely 5FU, ascorbic acid, paracetamol, pyridoxine, and streptomycin, do not demonstrate any inhibitory activity towards these enzymes. Based on the ADME analysis, it is observed that among the compounds studied, only Amlodipine exhibits inhibitory activity towards both CYP3A4 and CYP1A2 enzymes. In contrast, the other compounds, namely 5FU, ascorbic acid, paracetamol, pyridoxine, and streptomycin, do not demonstrate any inhibitory activity towards these enzymes. These findings suggest that a majority of the drugs on the listing will either have more therapeutic potential and may be metabolized in the liver. The bioavailability of indomethacin and diclofenac is comparatively higher than that of other drugs, with streptomycin being the last member in this category. The empirical evidence strongly indicates that the majority of the compounds exhibit a high degree of compatibility to be used in the *in vitro* minimum inhibitory concentration (MIC) and other related investigations.

Using Swiss ADME, the radar images are generated in which the pink area indicates the ideal range across the following attributes: lipophilicity (XLOGP3 − 0.7 to + 5.0), size (MW <500 Daltons), solubility (log S ≤ 6), total polarity surface area (TPSA 20-130 Å), flexibility (maximum 9 bonds with rotatable properties), and saturation (fraction of carbons in sp3 hybridization ≤ 0.25)[32]. As per the radar images in (Fig. 4), only pyridoxine, promethazine, and vitamin C are well within the desired range for oral bioavailability. Whereas, 5FU, amlodipine, calaptin, celecoxib, diclofenac, hydroxychloroquine, indomethacin, and paracetamolhave just one parameter, which is deflecting (as shown in Fig. 4) from the above-mentioned attributes. Since the reference drug, streptomycin, doesn't obey the Lipinski rule by 3 parameters (Table 4) but is still referred to as an FDA-approved drug[33]. So, these 12 drugs, as predicted by the docking analysis, were further accessed *in vitro* MIC analysis.

The *in silico* toxicity parameters of the selected drugs were calculated by using usingProTox 3.0, Tox-Prediction tool. Table 5 represents the toxicity profile

Table 4 — *In silico* pharmacokinetics of the top 12 selected drugs after molecular docking studies

Drugs	ESOL (Log S)	GIA	BBB permeant	P-gp substrate	CYP3A4 inhibitor	CYP1A2 inhibitor	(iLOGP)	Bioavailability score	Lipinski rule
Amlodipine	3.76	High	No	Yes	Yes	Yes	3.17	0.55	Yes, 0 violation
Hydroxychloroquine	-3.91	High	Yes	No	No	Yes	3.58	0.55	Yes, 0 violation
5FU	-0.58	High	No	No	No	No	0.44	0.55	Yes, 0 violation
Indomethacin	-4.86	High	Yes	No	No	Yes	2.76	0.85	Yes, 0 violation
Ascorbic acid	0.23	High	No	No	No	No	0.39	0.56	Yes, 0 violation
Calaptin	-4.46	High	Yes	Yes	Yes	No	4.5	0.55	Yes, 0 violation
Diclofenac	-4.65	High	Yes	No	No	Yes	1.98	0.85	Yes, 0 violation
Paracetamol	-1.34	High	Yes	No	No	No	1.21	0.55	Yes, 0 violation
Pyridoxine	-1.34	High	No	No	No	No	0	0.55	Yes, 0 violation
Celecoxib	-4.57	High	No	No	No	Yes	2.56	0.55	Yes, 0 violation
Atorvastatin	-5.99	Low	No	Yes	Yes	No	3.81	0.56	Yes, 1 violation
Promethazine	-4.88	High	Yes	No	No	Yes	3.12	0.55	Yes, 0 violation
Streptomycin	2.18	Low	No	Yes	No	No	-0.06	0.17	No; 3 violations: MW>500, NorO>10, NHorOH>5

Table 5 — *In silico* evaluation of the potential toxicity of the 12 selected drugs

Drugs	Predicted LD_{50}	Predicted Toxicity Class	Hepatotoxicity (DILI)	Nephrotoxicity	Cardiotoxicity
Amlodipine	37 mg/kg	2	Inactive	Active	Inactive
Hydroxychloroquine	1240 mg/kg	4	Inactive	Inactive	Inactive
5FU	1923 mg/kg	4	Inactive	Inactive	Inactive
Indomethacin	12 mg/kg	2	Active	Active	Inactive
Ascorbic Acid	3367 mg/kg	5	Inactive	Active	Inactive
Calaptin	108 mg/kg	3	Inactive	Inactive	Inactive
Diclofenac	53 mg/kg	3	Active	Active	Active
Paracetamol (Acetaminophen)	338 mg/kg	4	Active	Active	Inactive
Pyridoxine	3350 mg/kg	5	Inactive	Active	Inactive
Celecoxib	1400 mg/kg	4	Inactive	Inactive	Inactive
Atorvastatin	5000 mg/kg	5	Active	Active	Inactive
Promethazine	255 mg/kg	3	Inactive	Inactive	Inactive
Streptomycin	500 mg/kg	3	Inactive	Active	Inactive

of the top 12 selected drugs after the molecular docking study; calculations of LD_{50}, toxicity class prediction, and organ toxicity profile have been included. As per the analysis of the results, it was revealed that ascorbic acid, pyridoxine, and atorvastatin were the least toxic, having LD_{50} (median lethal dose) values of 3367mg/kg, 3350mg/kg, and 5000 mg/kg, respectively. Where LD_{50} represents the dose of a substance required to kill half of the members of a tested population treated over a specific period. All the above-mentioned three drugs belong to class 5 of the toxicity class, predicting that it is unlikely to cause any harm under normal circumstances of exposure. In contrast, amlodipine and indomethacin belonging to class 2 suggest their toxicity level to the bacterial strains, as organ toxicity of the drugs suggests amlodipine is nontoxic to the liver and heart, whereas indomethacin is inactive for heart toxicity. Some notable drugs such as hydroxychloroquine, 5FU, Calaptin, Celecoxib, and

Promethazine have shown a non-toxic behavior in relation to organ toxicity where all the drugs were inactive for the liver, kidney, and heart.

Minimum inhibitory concentration (MIC) analysis: MDR-resistant *Pseudomonas* spp.

The antibiotic and non-antibiotic drugs used in this study for repurposing were subjected to MIC testing against the different types of *Pseudomonas* spp. strain of clinical isolates (Table 6). Antibacterial susceptibility tests were done to establish the minimal inhibitory concentration. Isolated bacterial colonies were selected from an agar plate, put into Luria broth medium, and allowed to grow overnight. The bacterial suspension was changed to match McFarland's criterion of 0.5. By dilution with medium, the concentration of cells was brought to 1×10^6 cfu/mL. The antibacterial properties of non-antibiotic drugs were examined by utilizing the broth microdilution technique. DMSO was used to dissolve drugs. The chemical was produced from stock solution at concentrations of 0 µg/mL, 3.12 µg/mL, 6.25 µg/mL, 12.5 µg/mL, 25 µg/mL, 50 µg/mL, 100 µg/mL, and 200 µg/mL. Streptomycin was taken as a standard antibiotic.

A volume of 5 µL of test compound and 5 µL of cells were added to 190 µL of LB media in a 96-well plate, making a total of 200 µL in each well and followed by 24-h incubation. After 24 h incubation, resazurine dye solution was added to determine the cell viability. A volume of 20 µL of Resazurine was added to every well and incubated for 2 h. A dark purple color indicated the presence of dead cells, whereas a pink color indicated the presence of viable cells[34]. Absorbance was read at 570 nm and 590 nm using a microplate reader. The lowest concentration that exhibited an absence of viable cells seen as blue in color in resazurine assay was determined as minimum inhibitory concentration (MIC). Cell viability was calculated and expressed as a Percentage. From these MICs, sub-inhibitory but clinically relevant concentrations of the nonantibiotic drugs were chosen to assess the inhibitory effect of drugs against these microorganisms. In this study, for *in vitro* MIC assays, amlodipine, hydroxychloroquine, 5FU, Indomethacin, ascorbic acid, and calaptin were found to be more potent against all the strains among all the drugs.

Antibacterial activity results of this study demonstrate that all the identified drugs have antibacterial potential. Amlodipine showed the MIC, 7.87 -17.80 µg/mL. Akinjogunla *et al*. 2021 reported that amlodipine exhibited MIC in the range of 12.5-50 µg/mL against *P. aeruginosa*[35]. In our study, MIC of hydroxychloroquine was investigated in the range of 33 to 65 µg/mL, which is very effective as compared to a previously reported study which reported the MIC against *Pseudomonas* was in the range of 650 to 2500 µg/mL[36]. Diclofenac and indomethacin showed MIC in the range of 85.79-187.70 µg/mL and 34.23-110 µg/mL against *Pseudomonas*, respectively. Ahmed *et al*. 2017 reported that indomethacin showed the highest MIC against *P. aeruginosa* at 1024 µg/mL, which is very high in comparison to our study, whereas as per their study, diclofenac showed potent antimicrobial activity against *Pseudomonas* at MIC 256 µg/mL[37]. The activity of 5FU on *P. aeruginosa* was proximally studied by Di Bonaventura, Giovanni, *et al* 2022[38] showed susceptibility to the tested agent at MIC: 128–1024 µg/mL, which is much higher than the results of this study (8.60-60.01 µg/mL). In another study conducted by Abdelraheem W *et al*. 2022[39]

Table 6 — MIC calculations with the top 12 selected drugs with different MDR bacterial strains

		MIC values (µg/mL)			
Sr.no.	Drugs	*P. aeruginosa*	*P. stutzeri*	*P. fluroscence*	*P. ptuida*
1	Amlodipine	9.62	7.87	17.80	13.65
2	Hydroxychloroquine	65.43	33.94	65.161	44.817
3	5FU	8.60	16.05	45.56	60.01
4	Indomethacin	34.23	42.79	86.189	110.01
5	Ascorbic acid	30.65	60.41	22.01	32.74
6	Calaptin	56.70	62.67	90.44	116.93
7	Diclofenac	85.79	92.11	184.0	187.70
8	Paracetamol	56.70	190.70	86.84	193.5
9	Pyridoxine	197	88.24	189.4	185.7
10	Celecoxib	210.0	223.5	141.5	167.0
11	Atorvastatin	200.01	116.92	90.44	190.0
12	Promethazine	60.40	124.46	92.11	186.0

investigated the MIC of ascorbic acid against *P. aeruginosa* isolates in the range of 156 to 1250 µg/mL, whereas as per the findings of the current study, the MIC obtained was in the range of 22-60 µg/mL. In this study, celecoxib showed antimicrobial activity at MIC more than 200 µg/mL, and as per the study conducted by Annamanedi *et al* 2017, it was demonstrated that a 10-15% reduction in CFU/mL was observed in gram-negative strains on ampicillin (2 µg/mL) and celecoxib (3.82 µg/mL) containing agar plates when compared to ampicillin (2 µg/mL) alone containing agar plate[40]. Thangamani *et al*. 2015 it was shown that with MICs ranging from 8 to 32 µg/mL, celecoxib demonstrated antimicrobial action against all Gram-negative pathogens tested, including *P. aeruginosa, E. coli, K. pneumonia, S. Typhimurium, and A. baumannii*[41]. In the study conducted by Masadeh *et al*. 2012, the antibacterial activity of atorvastatin was investigated against different bacterial strains, including *Pseudomonas*, and findings showed that statins can elicit varying levels of antimicrobial activity, with atorvastatin being the most effective with MIC of 83.33 ± 36.08 to 95.83 ± 22.09 µg/mL[42] and these findings are close to current study with MIC of around 100 µg/mL.When treating infectious disorders, dietary supplements like ascorbic acid may sometimes be administered in addition to medicines. Studying their antibacterial activity and impact on bacterial antibiotic sensitivity is crucial, as shown by Abbas *et al*. 2012[43]. Ascorbic acid showed direct antibacterial activity in the Abbas *et al*. 2012[43] investigation. At 2 mg/mL, it was able to stop *P. aeruginosa* from growing while also having bactericidal effects at concentrations of 2 to 8 mg/mL. The minimum inhibitory concentrations for 18 out of the 20 *P. aeruginosa* isolates studied were found to be 10 to 40 mg/mL, according to Zhao and Liu6[34], and 20 to 50 mg/mL of NAC was found to be bactericidal for *P. aeruginosa*, according to Roberts and Cole[44] and these findings are quite higher than to our current study with MIC of 25-50 µg/mL.

Rhamnolipid Production Analysis

The rhamnolipid and phycocyanin production was estimated to check whether the selected drugs after the docking (drugs selected to block uS12 ribosomal protein: target to inhibit translation of proteins) and MIC study. Rhamnolipids are glycolipids containing one (mono-rhamnolipid) or two (di-rhamnolipid) l-rhamnose molecules. Rhamnosyltransferase 1 (RhlA and RhlB) catalyzes the synthesis of rhamnolipid[45]. The results of Figure 5A suggested positive protection for all the *Pseudomonas* spp. Strains. Where the control samples of *P. aeruginosa* yielded the highest levels in the range of 22.9 mg/mL with *P. putida* showing a very low production of just 0.005 mg/mL. Rhamnolipids are generally considered as one of the virulence factors for increasing the pathogenicity of the strains. The individual control strain production suggests the pathogenicity in the descending order of *P. aeruginosa* > *P. Stutzeri*> *P. fluorescence* > *P. Putida*. Drug treatments to the strains significantly reduce rhamnolipid production across strains. Indomethacin caused the most substantial decrease in production to 16.02 mg/mL from 22.9 mg/mL, while hydroxychloroquine caused the least 20.01 mg/mL in *P. aeruginosa*. For *P. stutzeri*, reductions ranged from 2.54 mg/mL with ascorbic acid to 1.54 mg/mL with hydroxychloroquine. *P. fluorescence* showed reductions between 0.7 and 1.24 mg/mL, with 5FU

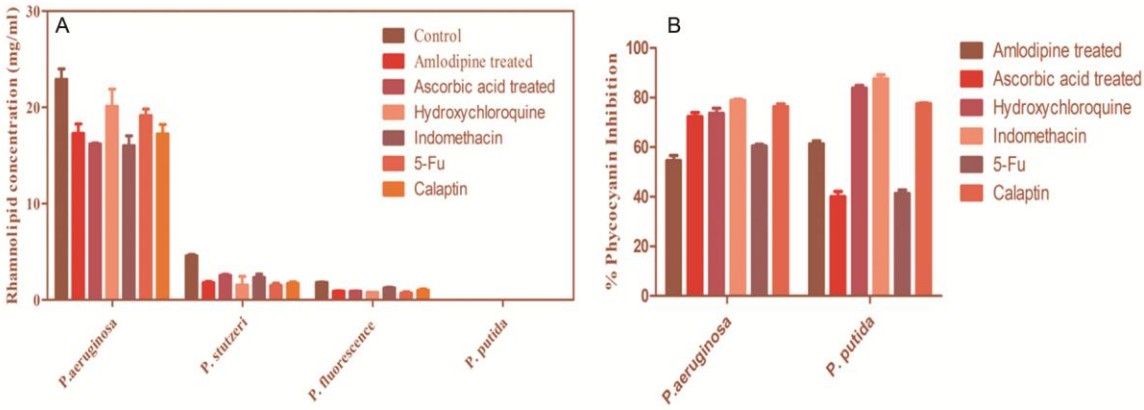

Fig. 5 — Estimation of virulence factor from *Pseudomonas* spp. Strains: (A) Rhamnolipid Production assay (The production from *P. putida* was very low (range of 0.001-0.005 mg/mL) as compared to others, so the bars are not visible in the graph; and (B) Phycocyanin inhibition assay

causing the greatest decrease. Amlodipine reduced rhamnolipid production in *P. putida* by up to 0.001 mg/mL, whereas, hydroxychloroquine and calaptin reduced it by at least 0.004 mg/mL.

Phycocyanin Inhibition Assay

Phycocyanin has emerged as a significant virulence component of *P. aeruginosa*. The level of phycocyanin production was evaluated with and without the drug. The test was used to compare the effects of the selected drugs on bacteria that were treated with MIC doses and the respective non-treated strains serving as control. The results are shown in Figure 5Bas the mean standard error of percentage change from the untreated controls of respective *P. aeruginosa* and *P. putida* strains. The analysis suggested that *P. fluorescence* and *P. stutzeri* were negative for the phycocyanin production inferring no phycocyanin production in both these strains. However, all selected drugs significantly reduced phycocyanin production in *P. aeruginosa and P. putida*. Identified drugs have demonstrated a noteworthy potential to inhibit phycocyanin synthesis and successfully limit phycocyanin pigment production. The experimental study revealed that the percentages of inhibition of pyocyanin pigment for *P. aeruginosa* range from 54.54% to 78.89%, whereas for *P. putida*, the range was 40 to 87.5%. The highest percentages of pyocyanin pigment inhibition were obtained in the presence of indomethacin, which was 78.89% and 87.5% for *P. aeruginosa and P. putida*, respectively, whereas, 5FU had the lowest phycocyanin inhibition percentage among all identified drugs, which was 60.42% and 41.25% for *P. aeruginosa and P. putida*, respectively.

Conclusion

Computer-aided drug repurposing is a potential approach to screen out the best candidates (bioactives/drugs) for the treatment of disease. Our findings confirmed the top 12 drugs with the high binding affinity (in reference to a control drug, streptomycin) out of the 63 drugs initially taken for *in silico* studies on uS12 ribosomal protein. Streptomycin works by inhibiting the action of the uS12 protein, which is a component protein of the 30s ribosome of prokaryotic cells. *In silico* pharmacokinetics and Lipinski rule of 5 revealed some drugs, out of the selected 12, didn't obtain attributes within the optimized limit. Since oral bioavailability can be either bypassed with intravenous forms or previous studies also report that the requirement to hold all the attributes in the optimized limit is not an obligate requirement for oral delivery. Thereby, all the top 12 best matches were used to calculate the MIC on *Pseudomonas* strains. Our result concluded that amlodipine, hydroxychloroquine, 5FU, Indomethacin, ascorbic acid, and calaptin were found to be more potent against all the strains. This study can be a great help in the treatment option of MDR resistance strain for UTI infections, mainly when the *Pseudomonas* species become resistant to the first line of antibiotics.

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Conflict of Interest

All authors declare no conflict of interest.

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