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EFFLUX PUMP INHIBITORY POTENTIAL OF CURCUMIN IN MDR *ESHCHERICHA COLI* CLINICAL ISOLATES COLLECTED FROM BANGLADESH

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ABSTRACT

Effluxing of antibiotic molecules out of the bacterial cell is the potential mechanism of developing resistance against diverse classes of antibiotics. Therefore, inhibition of these efflux pumps by using different efflux pump inhibitors (EPIs) may restore the efficacy of large number of antibiotics. Curcumin, a natural yellow pigment commonly found in turmeric, has been evaluated for its role as EPI in clinical isolates. Disc diffusion and broth dilution method were applied to evaluate the antimicrobial susceptibility whereas ethidium bromide-agar cartwheel method was used to evaluate the efflux pump activity of the 16 MDR clinical isolates of *E. coli*. Molecular docking studies of both the '*enol*' and '*keto*' structure of curcumin (due to tautomerism) and reference standards were carried out by using Autodock vina algorithm against different efflux pump proteins. Antimicrobial susceptibility tests showed high resistance to cefixime, linezolid, and chloramphenicol, among other antibiotics. Around 37.5% of the clinical isolates showed enhanced efflux pump activity compared to *S. aureus* ATCC 25922. Investigation into curcumin showed that it reduced the MIC of linezolid, and ciprofloxacin in efflux pump active isolates by 2-8 folds with a fractional inhibitory capacity ranging from 0.156-1.062. The results suggested that concomitant use of curcumin with ciprofloxacin and linezolid showed synergistic and additive effects. Reserpine was used as a reference standard efflux pump inhibitor, reducing the MIC of linezolid and ciprofloxacin among those same strains by 2-8 folds. The docking studies showed that the binding affinities of *'enol'* form of curcumin were found -7.3, -7.7, -7.5 and -7.1 Kcal/mol for AcrB, EmrD, MacA and MdfA efflux pump proteins, respectively. The result showed that curcumin may show synergistic or additive effects when used with current antibiotics by inhibiting ABC, MFS and RND efflux pump family. Further studies are recommended to understand the molecular mechanism of this activity by curcumin.

Keywords: Curcumin, efflux pump inhibitor, antibiotic resistance, clinical isolates, molecular docking, 96-well plate, ethidium bromide

INTRODUCTION

Antibiotic resistance is closely associated with the clinical and veterinary use of antibiotics. Prolong and uncontrolled use of antibiotic in clinical setting is highly associated with the development of antibiotic resistance. Control of irrational use of antibiotic is very difficult. For example, in hospitals, antibiotics are released into the sewage system without any prior treatment, leading to the development of resistant organisms. Also, the indiscriminate self-medication of the antibiotics by procuring them over the counter and using them in a sub-therapeutic dose is responsible for the development of antibiotic resistance (**Salam *et al.*, 2023**). According to the Centers for Disease Control and Prevention (CDC), USA, 30% of the prescribed antibiotics to the outpatients are unnecessary (**Sharma *et al.*, 2019**). The irresponsible use of antibiotics as a growth enhancer in farming also worsens the problem. The seriousness of this issue can easily be understood from the fact that in USA about 2 million people get infected by drug resistant bacteria every year among them thousand dies (**Haque *et al.*, 2018**).

Antibiotic resistance can be developed by various ways. There are two major genetic strategies by which bacteria adapt the resilience; a) Gene mutations often associated with the mechanism of action of the compound, b) accession of foreign DNA which codes for resistance determinants through horizontal gene transfer. Chemical alterations, destruction, decrease in concentration and efflux pumping of the antibiotic molecule can also develop resistance. Bacterial target site protection through modification, alteration through enzymatic activity, and total replacement may also impart antibiotic resistance against a variety of antibiotics (**Tajer *et al.*, 2024**).

Now in the era of antibiotics, efflux pumps are very important for bacterial survival and many show their resistance by overexpression of efflux pump proteins. As a result, they become the attractive target molecules for fighting against antibiotic resistance (**Gaurav *et al.*, 2023; Spengler *et al.*, 2017**). The primary role of these pumps is to expel the toxic materials outside of the bacterial cell and causes resistance against various antibiotics including β lactam, carbapenems, fluoroquinolones, protein synthesis inhibitors and polymyxins etc (**Munita *et al.*, 2016**). In humans, they are responsible for bacterial resistance and the failure of chemotherapy; most of them belong to the ATP-Binding Cassette (ABC) superfamily (**Eckford *et al.*, 2009**). Bacteria contains wide range of efflux pump systems. NorA, MexAB-OprM, and AcrAB-TolC systems were widely studied and contribute highly to the development of bacterial resistance (**Nishino *et al.*, 2021**). Inhibition of efflux pump can potentiate the activity of several antibiotics. Considering this, the first inhibitor of EP was reserpine, a plant alkaloid used to treat hypertension (**Seukep *et al.*, 2020**). Several classes of plant derived secondary metabolites such as polyphenols, diterpenes, oligosaccharides, alkaloids were explored for the EP inhibitory potentials in different bacteria (**Zhang *et al.*, 2023**). Curcumin is a phenolic compound obtained from the rhizomes of the medicinal plant *Curcuma longa* (Fam: Zingiberaceae) commonly known as turmeric. Isolated curcumin has been shown to exhibit antimicrobial properties through anti-biofilm, anti-quorum sensing and inhibition of bacterial efflux pumps (**Adamczak *et al.*, 2020**). Moreover, **Jaberi *et al.* (2018)** reported the norA efflux pump expression inhibition in *S. aureus* using curcumin. Not only that, curcumin can bind with hsp90 and inhibit efflux pump of a pathogenic fungus, *Candida albicans* (**Lee *et al.*, 2022**). Water soluble galactose-clicked curcumin in adjuvant therapy with meropenem showed excellent antibacterial and antiefflux activity towards *Klebsiella pneumoniae* through binding with AcrAB-TolC efflux pump systems (**Yadab *et al.*, 2021**). The same pump is also effective in *E. coli* for developing multidrug resistance against variety of antibiotics (**Smith *et al.*, 2024**).

Therefore, the study aims to evaluate curcumin's anti efflux activity against *E. coli* clinical isolates. The *in-vitro* and *in-silico* study approach is considered to investigate the possible role of curcumin in overcoming the efflux pump-mediated resistance.

MATERIALS AND METHODS

Chemicals

Curcumin [Pubchem CID: 969516] was purchased from Tokyo Chemical Industries, Japan. Ethidium bromide and reserpine was brought from Sigma Chemical Co. (St. Louis, MO, USA). Resazurin sodium salt dye was purchased from Loba Chemicals India. Muller-Hinton agar (MHA), Muller-Hinton broth (MHB), Tryptic soybroth (TSB), nutrient agar (NA), nutrient broth media (NB) were purchased from TM media Ltd. India. Standard antibiotic powders were purchased from Advance Chemical Industries Ltd. Dhaka, Bangladesh. Different kinds of antibiotic discs were purchased from Himedia (India) and blank was prepared by perforating sterile Whatman filter paper No. 1 carefully.

Collection and preservation of clinical isolates

The pure cultures of the clinical isolates of *E. coli* were collected from a diagnostic center namely Popular Diagnostic Center, Dhanmondi branch, Dhaka in a sterile test tube filled with Tryptic soy broth under Laminar Airflow (LAF) bench. The isolates were identified according to the established laboratory protocol. After inoculation of the pure cultures, the test tubes were immediately transferred to the laboratory and incubated into the shaker incubator at 220 rpm and 35±2ºC for two hours. Then, agar slants were prepared in a screw cap test tube with sterile tryptic soy agar (30gm of TSB powder and 15gm of Agar was dissolved in distilled water to prepare 1L solution). Specific clinical isolates of the bacteria were inoculated into each slant and then the slants were incubated in an incubator at 35±2ºC for 24 hours.

Antibacterial susceptibility test by Kirby-Bauer disc diffusion method

A popular method named Kirby-Bauer disc diffusion technique was applied to understand the susceptibility of the clinical isolates towards different antibiotics (**Suma *et al.*, 2023**). A total of seven antibiotics such as ciprofloxacin, tetracycline, cefixime, amikacin, linezolid, azithromycin, chloramphenicol was selected to determine their resistance profile against collected clinical isolates. To conduct the susceptibility test, Mueller-Hinton Broth (MHB) solution and MHA agar were prepared and sterilized by following appropriate guidelines (CLSI). 2ml of non-sterile MHB is placed into each test tube and were autoclaved. The clinical isolates preserved into TSB were inoculated into the test tube containing MHB using aseptic technique under the laminar air flow and incubated at 37°C for approximately 2 hours and the growth of the bacteria was adjusted to that of 0.5 McFarland standard (10^5 CFU/mL) through dilution with sterile MHB. Sterile MHA was poured into the sterile petri dishes (150mm diameter) to get the thickness of 5.0 mm. The sterility of MHA media in the petri dishes was confirmed by incubation for 24 hours under 37°C. Freshly cultured bacteria in MHB were inoculated into the petri dishes by sterile cotton swab. The antibiotic discs were placed in the plates with a sterile forceps and the plates were incubated at 37 ^{0}C for 18-24 hours. The clear (transparent) zone of inhibition around the discs were measured using a ruler graduated in millimeters.

Minimum Inhibitory Concentration (MIC) determination

Broth microdilution technique was used to determine MIC level of the seven antibiotics (**Wiegand *et al.*, 2008**). Sterile solution of cation adjusted MHB was used to make freshly prepared bacterial concentration to 0.5 McFarland standard by standard protocol. This solution was further diluted 100 times by adding 100µl of the adjusted bacterial culture to 9.9ml of CAMHB (1×10^5 CFU/mL). Under laminar air flow a sterile 96 well plate with a lid was opened and 100µl of CAMHB bacterial culture along with 90 µL of CAMHB were added to each well of the plate by using the 8-channel micropipette. Negative control was prepared by adding broth media only and vehicle only. 10µL of the antibiotic solution of desired concentration was added to each well of the first row, except the positive control row. Every 2 adjacent columns contained a single antibiotic. The antibiotics are diluted 2-fold for up to 10 times starting from 512 to 1 µg/mL. The plate is incubated for 18- 20 h and after incubation period, previously prepared 20 µL of resazurin sodium salt dye (20 µg/mL-0.02%) was added and incubated for one hour and observed the color changes from blue to orange in naked eye if there is any viable cell. Following incubation, the MIC was visually determined as the lowest dose at which colour development was inhibited.

Efflux pump activity study of the clinical isolates

Slightly modified ethidium bromide-agar cartwheel method (**Munira *et al.*, 2022**) was used to determine the efflux pump activity in these clinical isolates. The bacterial solutions in TSB media were prepared and bacterial concentration was adjusted to 0.5 McFarland Standard (KB test). 100µg/mL stock solution of Et-Br was prepared and sterilized by filtration using a syringe filter (0.22µm pore size). TSA plates with various concentrations of Et-Br such as 0.5µg/mL, 1.0µg/mL, 1.5µg/mL, 2.0µg/mL, and 2.5µg/mL were prepared as such that the final volume of the agar was 50ml. The bacterial cultures were then swabbed on the EtBr-TSA plates. Each plate should include at least one reference strain (SA ATCC25923), that serve as a control for fluorescence analysis. After that the clinical isolates were inoculated into each of the plate in a cartwheel pattern, were incubated at 35±2ºC for 18-24 hours and were observed at 365nm wavelength UV transilluminator. The minimum concentration of EtBr that produces fluorescence of the bacterial mass was recorded. Overexpressing of efflux pump activity in the isolates shows fluorescence in significantly higher concentrations than the reference strain. Efflux pump activity index is determined using the following formula:

Efflux pump activity index = MC_{EtBr} (MDR) – MC_{EtBr} (Ref)/ MC_{EtBr} (Ref)

Here, MC_{EtBr}(MDR) is the minimum concentration of EtBr at which the growth of a clinical isolate shows fluorescence, and MC_{EtBr}(REF) is the minimum concentration of EtBr at which the reference strain shows fluorescence (**Martins *et al.*, 2011**).

EPI activity of curcumin and reserpine using broth microdilution method

The isolates that have shown increase efflux activity in the Et-Br agar cartwheel experiment were subjected to study the effect of curcumin on reducing the MIC values of antibiotics. It is to be noted that EPI activity can be discerned if curcumin along with antibiotic combination can reduce the MIC values which is required for an antibiotic. 128 µg/mL of curcumin stock solution was prepared using 10% v/v ethanol-water as the solvent and reserpine was used as standard (32 µg/mL). CAMHB (Cation Adjusted Muller Hinton Broth) media was used for bacterial solution preparation and the concentration was adjusted to McFarland standard 0.5 and then 100µL solutions were added to each well of 96 well plates. Then, 10 µL of antibiotic solution was added to each well and also reduced the concentration of the antibiotic to half to each well. the final concentration of the antibiotic was started from 1/8th of the stock concentration and geometrically decreased 2-folds up to 1/1024th of stock concentration. Additional 10 µL test EPI solution of curcumin and reserpine was added to the well, respectively to observe their activity. After preparation of the microplate then it was incubated for 18-24 hours in a shaker incubator at 110 rpm and 35±2ºC. After incubation the plate were examined in a microplate reader at 595nm. The FIC values for each component (A and B) were calculated using the following equations where A represents the test sample and B represents the conventional antibiotic.

FIC (A) = MIC (Combination of A and B)/ MIC (A independently)

FIC (B) = MIC (Combination of B and A)/ MIC (B independently)

The ΣFIC was then calculated using the formula ΣFIC = FIC(A) + FIC(B). The interactions were classified as synergistic (ΣFIC ≤0.5), additive (ΣFIC >0.5-1.0), indifferent (ΣFIC >1.0-4.0) or antagonistic (ΣFIC >4.0) (**Wiegand *et al.*, 2008**).

Molecular docking studies

Molecular docking study is an easy tool to understand whether curcumin can bind with the bacterial efflux pump proteins or not. A three-dimensional (3D) homology models of AcrB, EmrD, MacA and MdfA were developed using Modeller software (version 9.25) with UCSF Chimera. For this purpose, sequence of AcrB, EmrD, MacA and MdfA were subjected to BLASTp analysis against the PDB database to identify a suitable template. *Escherichia coli* (PDB ID: 3WDO) was selected based on the BLAST score, E-value and percent identity (**Jiang *et al.*, 2013**). The homology model was subjected to energy minimization using Modeller (version 9.25). The quality of the homology model was checked using PROCHECK (**Laskowski *et al.*, 1993**), Verify 3D (**Luthy *et al.*, 1992**) and ERRAT (**Colovos *et al.*, 1993**). Molecular docking was carried out using Autodock Vina with PyRx. The structure of curcumin and reference compounds were drawn in Spartan 14. For each structure conformer distribution was calculated at ground state using the Merck Molecular Force Field (MMFF) model and top 100 confirmers (if more than 100 conformations were generated) of lowest energy were saved. Equilibrium geometry of each conformation was calculated by the semi-empirical method using the AM1 mathematical model. Only the conformation with the lowest energy was saved in sybyl mol2 format. This file was opened in Gaussian 09W and the structures were fully optimized by employing Density Functional Theory using Becke's exchange Functional combining Lee, Young, and Parr's correlation Functional (B3LYP). The basis set used was 6-31G*. The optimized structures were saved in the pdb format. The optimized structures were docked against the refined homology models of AcrB, EmrD, MacA and MdfA with an exhaustiveness of 8 and maximization of search space. The best results were saved and analyzed with Discovery Studio Visualizer (**Munira *et al.*, 2022**).

RESULT AND DISCUSSION

Besides culinary purposes, curcumin has shown a wide variety of biological activities like anticancer, antimicrobial, anti-inflammatory, anti-oxidant etc. (**Fu *et al.*, 2021**). It has already been reported that curcumin reduces MIC level of several antibiotics against multidrug resistance *Pseudomonas aeruginosa* (**Negi *et al.*, 2014**). Previous study reported that the concomitant use of curcumin with antibiotics can potentiate their activity in *S. aureus* (**Moghaddam *et al.*, 2009**). Present study deals with the investigation into overcoming efflux pump mediated

antibiotic resistance using curcumin concomitantly with linezolid and ciprofloxacin.

16 clinical isolates were collected from the local diagnostic centers and identified by following standard protocol. The Kirby-Bauer disc diffusion assay was conducted to evaluate their antibiotic susceptibility and the result (**Table 1**) delineates a picture of the resistance pattern among the *E. coli* samples against different antibiotics. All the isolates showed 100% resistance against linezolid and cefixime whereas 87.5% isolates were resistant to ciprofloxacin. These finding was in accordance with the previous report where 453 clinical isolates of *E. coli* collected from Bangladeshi UTI patients showed resistant to at least one antibiotic, and 92% were found resistant to 3 or more classes of antibiotics, thus classified as MDR *E. coli* (**Nobel *et al.*, 2021**). However, 87.5% isolates were sensitive to chloramphenicol with MIC value ranging from 2-8 µg/mL. **Moniruzzaman *et al.* (2023)** reported the similar findings where chloramphenicol was found sensitive in 88.9% of the multidrug resistant *E. coli* samples. MIC tests with the same antibiotics were also evaluated to determine the lowest concentration of antibiotics required for growth inhibition, as represented in Table 1. The MIC breakpoints for each antibiotic are evaluated based on the recommendation chart of BSAC, UK (**Andrews, 2008**). MIC values of cefixime, linezolid, ciprofloxacin and tetracycline were found in a wide range between >256, 128-256, 32-256 and 64-256 µg/mL, respectively which indicated these clinical isolates are multidrug resistant and completely resistant to at least two of these antibiotics. These findings are in accordance with previous report on MIC values of different antibiotics on resistant *E. coli* clinical isolates (**Sultana *et al.*, 2018**).

Table 1 Antibiotic sensitivity pattern of *E. coli* strains against different antibiotics (n=16)

Antibiotic disc	Resistant (%)	Intermediate (%)	Sensitive (%)	MIC value (µg/mL)
Tetracycline	75.0	6.25	18.75	64-256
Chloramphenicol	6.25	6.25	87.5	2 - 8
Linezolid	100	0.0	0.0	128 - 256
Ciprofloxacin	87.5	6.25	6.25	32 - 256
Amikacin	50	25.0	25.0	4 - 128
Azithromycin	75.0	18.75	6.25	2 - 32
Cefixime	100	0.0	0.0	>256

Membrane bound proteins can efflux various chemicals from the inside the cell towards outside, which is called efflux process using either an ion gradient (commonly H^+) or ATP cleavage to provide the required energy (**Poole, 2005**). Ethidium bromide can intercalate between DNA strands and emits fluorescence which can be detected by UV transluminator. If the isolates have active efflux proteins, fluorescence will not be detected (**Martins *et al.*, 2013**). So, using the ethidium bromide-agar cartwheel method, the presence of efflux pump activity in the clinical isolates was assessed; the results were summarized in table 2 as the efflux pump activity index. Six isolates among the collected samples showed efflux pump activity index more than 2.0 which implied that the isolates had active efflux membrane proteins (**Table 2**). Previous study showed that *E. coli* isolates overexpressed AcrAB-TolC efflux pump activity due to their intrinsic resistance as well as up-regulating expression of efflux pumps because of environmental stressor during adaptive resistance (**Sharma *et al.*, 2019**). **Chowdhury *et al.* (2019)** reported that 77% of the clinical isolates of *E. coli* possessed efflux pump activity against three or more antibiotics using the same method which is in accordance with our result too.

Among the six efflux pump active isolates, MIC value of linezolid and ciprofloxacin were found substantially higher than other antibiotics. The concomitant use of curcumin along with these antibiotics reduced the required MIC value which was evaluated by broth dilution technique. When the antibiotic linezolid and curcumin was used alone, MIC value was found at >128 and >256 µg/mL, respectively. The concomitant use of curcumin with the antibiotic at a dose of 128 µg/mL reduced the MIC value of the antibiotic to 32-64 µg/mL. The calculated fractional inhibitory capacity (ΣFIC) values of curcumin with linezolid were found in the range of 0.3755 to 0.75 among efflux pump active isolates (**Table 3**).

Table 2 Efflux pump activity of different clinical samples of *E. coli* (n=6)

Identifier	MC_{EtBr} (µg/mL)	Index
SA ATCC25923*	0.5	0
EC 298566UKP	>2.5	>4.0
EC 2184160P	>2.5	>4.0
EC39658M	2.5	4.0
EC83926M	2.5	4.0
EC 38247M	2.0	3.0
EC33014188P	1.5	2.0

* Reference standard

Besides, ΣFIC values of curcumin with ciprofloxacin were found in a range of 0.156-1.062 (**Table 4**). Reserpine was used as standard control in both cases which displayed ΣFIC values ranging from 0.312-1.25 and 0.5-0.75 along with linezolid and ciprofloxacin in the same isolates. Compared to positive standard, it is evident that curcumin reduced the MIC value of linezolid to 2-4 folds, showing potential synergistic activity in two isolates and additive activity in other isolates. Previously published review report on the antibacterial activity of curcumin showed that the coadministration of curcumin potentiate the activity of several antibiotics like ciprofloxacin, tetracycline, colistin etc (**Dai *et al.*, 2022**). However, none of the studies reported the synergism of curcumin with linezolid. So, this is the first report on the synergistic action of curcumin with linezolid on the *E. coli* isolates.

Table 3 Con-comitant use of curcumin and linezolid

Clinical isolates	MIC of linezolid (µg/mL)	MIC of curcumin (µg/mL)	MIC of antibiotic + curcumin (µg/mL)	Σ FIC value	MIC of reserpine (µg/mL)	MIC of reserpine + linezolid (µg/mL)	Σ FIC value
EC 298566UKP	>128	>256	64	0.75	32	16	0.625
EC 2184160P	>128	>256	64	0.75	64	16	0.375
EC39658M	>128	>256	64	0.75	64	64	1.25
EC83926M	>128	>256	64	0.75	32	8	0.312
EC 38247M	>128	>256	32	0.375	32	8	0.312
EC33014188P	>128	>256	32	0.375	32	16	0.625

Table 4 Con-comitant use of curcumin and ciprofloxacin

Clinical isolates	MIC of ciprofloxacin (µg/mL)	MIC of curcumin (µg/mL)	MIC of antibiotic + curcumin (µg/mL)	Σ FIC value	MIC of reserpine (µg/mL)	MIC of reserpine + ciprofloxacin (µg/mL)	Σ FIC value
EC 298566UKP	>256	>256	64	0.5	32	16	0.562
EC 2184160P	>128	>256	64	0.75	64	32	0.75
EC39658M	>128	>256	64	0.75	64	32	0.75
EC83926M	>32	>256	16	0.562	32	8	0.5
EC 38247M	>32	>256	32	1.062	32	8	0.5
EC33014188P	>64	>256	16	0.156	32	16	0.75

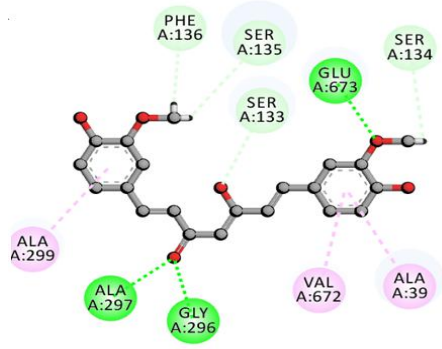
For a better understanding, molecular docking approach (*in silico*) was taken into consideration to evaluate the role of curcumin on different bacterial efflux pump proteins using suitable tools. Five kinds of efflux pumps such as ABC (ATP Binding Cassette) superfamily, MFS (Major Facilitator Super) family, MATE (Multidrug and Toxic Compound Extrusion) family, SMR (Small Multidrug Resistance) family and RND (Resistance Nodulation Division) family are found in the *Gram-negative* bacteria. Among them, the RND family protein AcrB is distributed throughout the transmembrane and periplasmic domain which is

responsible for extrusion of ethidium bromide, ciprofloxacin, β-lactams, tetracyclines etc (**Husain *et al.*, 2010**). EmrD, an MFS superfamily transporter protein, is responsible for expelling out amphipathic compounds across the inner membrane of *Escherichia coli* (**Yin *et al.*, 2006**). MacA (Macrolide export protein) efflux pump, belonging to the ABC superfamily, form a MacA-MacB-TolC tripartite system in *E. coli* that drives out macrolides and glycopeptides (**Fitzpatrick *et al.*, 2017**). *E. coli* transporters MdfA is a close homolog to EmrD. MdfA, a putative membrane protein of 410 amino acid, belongs to MFS superfamily. This pump confers resistance to clinically important antibiotics such as chloramphenicol, erythromycin, certain aminoglycosides, and fluoroquinolones (**Edgar *et al.*, 1997**). For this study, based on the crystal structure of the AcrB, EmrD, MacA and MdfA from *E. coli* were obtained from the protein data bank PDB ID: 2j8s, 3fpp, 2GFP and 4ZP0, respectively. The suitable models were constructed using with SWISS-Model, Phyre2 and BLASTp analysis. Hydrogens were added to the models and the energy was optimized in lowest level. PROCHECK confirmed the quality of the models and environmental profile was obtained using Verify 3D-1D (all models scored ≥0.1) (Figure 1).

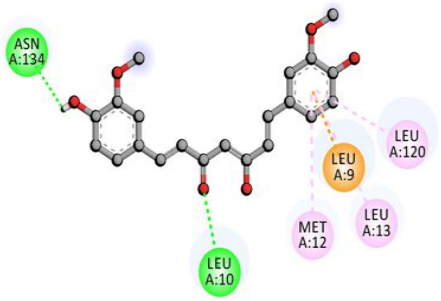
The molecular docking study's result shows that curcumin and reserpine had strong binding affinity towards efflux pump proteins (**Table 5**). It is evident that '*enol*' form of curcumin showed favorable binding affinity than '*keto*' form. Highest binding affinity was observed in EmrD and '*enol*' form of curcumin with a value of -7.7 kcal/mol whereas second highest binding affinity (-7.5 kcal/mol) was shown to MacA protein. Strong pi-aryl hydrophobic bonding was observed in case of '*enol*' form of curcumin to Ala299, Ala39 and Val672 aa. residues of AcrB protein (**Figure 1A**). Previous studies showed that the surface contacts were formed between efflux pump inhibitors and Phe136, Glu176, Gly179 residues of AcrB pump through hydrogen bonding (**Phan *et al.*, 2022**). The hydrophobic bonding was occurred in Leu9, Met12, Leu13 and Leu120 residues in addition of the same bonding with leu10 and Asn134 in EmrD protein (**Figure 1B**). Similar docking patterns were reported earlier with black pepper phytoconstituent against EmrD efflux pump (**Dongre *et al.*, 2022**). Several interactions allowed curcumin to show greater affinity towards EmrD. The binding interactions of '*enol*' form of curcumin with MacA reveals that the pi-alkyl hydrophobic bonding took place in Arg93 from both aryl rings and conventional hydrogen bondings were occurred in Leu96, Gln149 and Leu641 amino acid residues in MacA efflux pump protein (**Figure 1C**). Hydrophobic pi-alkyl bonding was observed with Pro74 and Ala128 residues whereas pi-sigma bonding with Gly347 during interactions with MdfA pump (**Figure 1D**). The same proteins showed hydrogen bonding in two amino acid residues namely Thr197 and Gly123 when docked with '*enol*' form of curcumin. Barberine, an isoquinoline alkaloid showed potential inhibition of MdfA efflux pump protein in *E. coli* through hydrophobic bonding at Leu41 to Val44 region and extended interactions were detected for regions of Glu132 to Glu135, Leu193 to Ile199, Ile247 to Gly249, and Trp372 to Gly376 residues (**Li *et al.*, 2023**). Reserpine showed better bonding interactions such as pi-alkyl, pi-aryl, pi-sigma, pi-pi T shaped hydrophobic bonding with various amino acid residues and conventional hydrogen bonding too (**Table 5**).

Table 5 Binding affinity calculations of the ligands with the efflux pump protein homology models

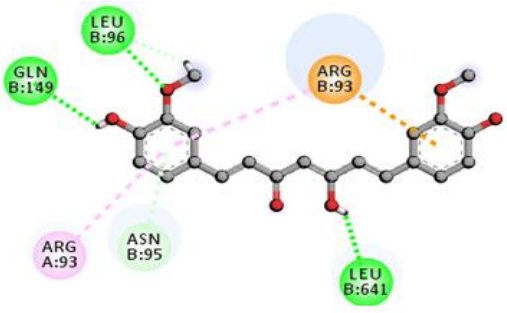
Efflux protein	Binding Affinity (kcal/mol)			Interacting Residues		
	Reserpine	Curcumin (Enol)	Curcumin (Keto)	Reserpine	Curcumin (Enol)	Curcumin (Keto)
AcrB	-8.8	-7.3	-6.4	Ala39, Ile38, Val672, Ala670, Phe136, Tyr327, Ser133, Ser134, Ser135	Ala39, Val672, Gly296, Ala297, Ala299, Glu673,Ser133, Ser134,Ser135, Phe136	Ala39, Val672, Glu673, Ser135, Ala297, Ala299, Gly296
EmrD	-9.5	-7.7	-6.8	Met12, Leu9, Leu10, Leu13, Leu120,Cys229, Gly42, Glu43, Asn143	Met12, Leu9, Leu10, Leu13, Leu120, Asn134	Met12, Leu10, Leu13, Leu120, Asn134
MacA	-8.3	-7.5	-6.7	Arg93, Gln149, Leu641, Leu96, Asn95, Ile590, Ile527, Phe594	Arg93, Asn95, Leu96, Gln149, Leu641	Arg93, Asn95, Leu96, Gln149
MdfA	-8.9	-7.1	-7.0	Pro74, Ala128, Lys346, Tyr127, Leu339, Tyr127, Lys346, Gly123	Pro74, Gly123, Ala128, Thr197, Lys346, Gly347, Thr348, Ser150	Pro74, Gly123, Ala128, Thr197, Lys346, Thr348, Ser150



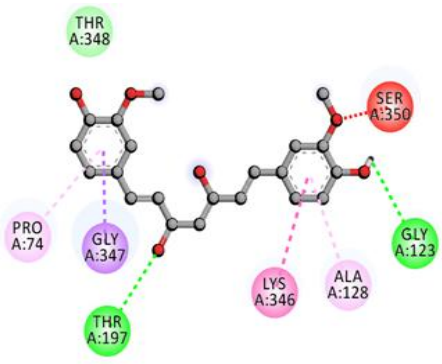
A: Interaction with AcrB



B: Interaction with EmrD



C: Interaction with MacA



D: Interaction with MdfA

Figure 1 Interaction between curcumin (enol) and different efflux pump proteins homology model

However, poor bioavailability, fast metabolism and chemical instability of curcumin are major challenges to achieving the desired effective plasma concentration (**Lopresti, 2018**). Therefore, potential binding with the efflux pumps for exhibiting synergistic activity of curcumin with available antibiotics may be greatly dwindled. This issue can be addressed by applying improvised formulation such as liposomal drug delivery, nanoparticle, complexation with phospholipids etc. which are already reported (**Hegde *et al.*, 2023**).

CONCLUSION

The multidrug efflux pump is one of the major causes of the development of antibacterial resistance in bacteria, which can be targeted with natural efflux pump inhibitors. Curcumin, a natural compound, has been considered for the present study for drug-resistance-reversal or re-sensitizing activities of linezolid and ciprofloxacin resistance in *E coli* clinical samples. The *in vitro* results showed promising antieffluxing activity of curcumin against efflux pump active isolates. The ∑FIC values of curcumin coadministered with these antibiotics has paved its efflux pump inhibitory potentials. The *in vitro* data was further reinforced by molecular docking approaches that showed strong binding affinity towards different efflux pumps namely AcrB, EmrD, MacA and MdfA homology models. Although the result suggested curcumin's potential role in reducing MIC values, it is very tough to unveil the exact molecular mechanism. Moreover, the use of curcumin has been compromised due to its poor bioavailability, which is the major limitation of this study. However, further studies are required to establish that co-administration of curcumin with antibiotics may ameliorate the present antibiotic resistance conditions.

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