Efflux Pump Inhibitory Potential of Vitexin 2"-*O*-xyloside Against Gram Positive Bacteria *Staphylococcus aureus*

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ABSTRACT: Expression of bacterial efflux pump is one of the major causes for developing antibiotic resistance in a variety of pathogens. Efflux pump inhibitors (EPIs) are potential molecules that can antagonize those pumps and help in alleviating the resistance problem. The study was aimed to explore the EPI activity of vitexin 2"-*O*-xyloside, an apigenin flavone glycoside. A total 15 clinical isolates of *Staphylococcus aureus* were collected and their antibiotic resistance profiles were detected by Kirby-Bauer disc diffusion assay and MIC values were determined through broth microdilution technique. Prevalence of efflux pump activity were examined through ethidium bromide agar-cartwheel method. Fractional inhibitory assay was carried out in combination with tetracycline and ciprofloxacin against clinical isolates of *S. aureus* with efflux pump activity. Finally, molecular docking approach was carried out in the active binding sites of NorA efflux pump protein by Autodock Vina. All of the clinical isolates showed resistance to cefixime, ciprofloxacin and tetracycline antibiotics whereas all of them were sensitive to chloramphenicol. Efflux pump was found active among 20% of the clinical isolates. The tested compound showed additive effective (ΣFIC value 0.625 – 0.75) when co-treated with tetracycline in the efflux pump active isolates which was similar to reserpine. Molecular docking studies showed that vitexin 2"-*O*-xyloside may bind to the different binding sites, opening the door for it to be considered as a potential EPI.

Key words: Vitexin 2"-*O*-xyloside, Efflux pump inhibitor, Antibiotic resistance, Clinical isolates, Molecular docking, 96-Well plate, Ethidium bromide

INTRODUCTION

Staphylococcus aureus, a Gram positive human pathogen is generally responsible for food borne diseases, upper respiratory tract infections, skin and soft tissue infections etc.[1] Infections caused by *S. aureus* can be hospital or community acquired which may lead to life threatening conditions like bacteremia, infective endocarditis, pneumonia, meningitis etc. Treatments become challenging with methicillin-resistant *S. aureus* (MRSA), vancomycin-intermediate-resistant *S. aureus* (VISA), and vancomycin-resistant *S. aureus* (VRSA) which are considered as superbugs and are enlisted in the '12 priority pathogens' by WHO due to their multidrug resistance.[2] The major mechanisms involved in the resistance of *S. aureus* against antibiotics are acquired by horizontal gene transfer of mobile genetic elements that causes modification of the antibiotic target, enzymatic inactivation of the antibiotic, decreased intracellular antibiotic concentration due to increased efflux.[3] One of the widely implicated mechanisms is the expelling of antibiotics out of the cells through efflux pumps.[4] Efflux pumps are transport proteins involved in the extrusion of toxic substrates from cells into the external environment. These proteins are found in both Gram-positive and Gram-negative bacteria as well as in eukaryotic organisms.[5]

Variety of efflux pumps have been found to be associated with the membrane of *S. aureus* which attributes to the drug resistance against penicillins, cephalosporins, fluoroquinolones, aminoglycosides etc.[6,7] Particularly in *S. aureus*, multi-drug resistance

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pumps are mainly conferred by Major Facilitator supefamily efflux systems, the most studied pumps being NorA, NorB, LmrS, MdeA, MefA, TetA etc.[8] It is to be noted that *S. aureus* is generally less susceptible to quinolones due to active NorA efflux pump in their membrane.[9] Several studies also showed that NorA pump may have ascertained the less susceptibility of this species towards other antibiotics too.[10] Continueous efforts have been given to search efflux pump inhibitors that can restore the activity of antibiotics by inhibiting the multi drug resistance (MDR) pumps. Kalia *et al.* (2012) has reported that capsaicin, major constituent from capsicum may be a potential molecule to be exploited as the inhibitor of NorA efflux pump in *S. aureus*.[11] Synthetic phenothiazine derivatives also showed promising activity as NorA efflux pump inhibitors.[12] Piperine from *Piper nigrum* and its analogs also showed inhibitory activity against NorA efflux pumps.[13]

The first discovered inhibitor of efflux pump (EP) in Gram-positive *Bacillus subtilis* was antihypertensive plant alkaloid reserpine.[14] Several classes of natural compounds have been described as EPIs, *e.g.* polyphenols, diterpenes, oligosaccharides, alkaloids.[15] Many natural products have been studied for their EPI activity, and some have been proven to show strong EPI activity like reserpine, capsaicin, genistein, silybin, orobol, biochanin, bonducellin, etc. Various flavonoids, *e.g.* chalcone and isoflavone were also studied for their EPI activity against NorA pump.[16] So it is logical to assume that vitexin 2''-*O*-xyloside, an apigenin flavone diglycoside, may also possess EPI activity. By reviewing the literature, no study on the activity of vitexin 2''-*O*-xyloside as a potential EPI was found. This work investigated the potential of vitexin 2''-*O*-xyloside as an inhibitor of EP by *in-vitro* and *in-silico* study.

METHOD AND MATERIALS

Chemicals. Vitexin 2''-*O*-xyloside was previously isolated from *Pothos scandens* and was reported earlier in another study.[17] Ethidium bromide and reserpine was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Resazurin sodium salt dye was purchased from Loba Chemicals India. Muller-Hinton agar, Muller-Hinton broth, Tryptic soybroth, nutrient agar, nutrient broth media were from TM media Ltd. India. Tetracycline, amikacin, chloramphenicol, azithromycin, cefixime, linezolid, ciprofloxacin standards were generously donated by Advance Chemical Industries Ltd. Dhaka, Bangladesh. Antibiotic discs were purchased from Himedia (India) and blank discs were prepared by perforating Whatman filter paper No. 1.

Bacterial culturing and antibacterial assay. All the experiments were done according to the Clinical Laboratory Standards Institute (CLSI) guidelines and the European Committee on Antibiotic Susceptibility Testing (EUCAST). Total 15 clinical isolates of *Staphylococcus aureus* were collected from Bangabandhu Sheikh Mujib Medical University, Bangladesh. Isolates were identified by the standard protocol developed by the center. Clinical isolates were copied to culture in different test tubes. The sterile tubes containing TSB were inoculated with the pure culture of the clinical isolates under laminar airflow (LAF) bench and were incubated at 37 ± 1° C for 18-20 h.

Antibacterial susceptibility test was performed by Kirby-Bauer disc diffusion technique.[18] Following appropriate guidelines, Mueller-Hinton broth (MHB) solution and Mueller-Hinton agar (MHA) were prepared and sterilized. Clinical isolates preserved in TSB were inoculated into the test tubes containing nutrient broth and were placed into shaker incubator for 2 h at a temperature of 37°C . The bacterial count was adjusted to that of 0.5 McFarland standard (10^5 CFU/ml) by adding phosphate buffer saline. Sterile MHA was poured into the sterile Petri dishes and after the sterility of the plates were confirmed, bacteria 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°C for 18-24 h. The clear (transparent) zones around the discs were measured.

Determination of minimum inhibitory concentration (MIC). Broth microdilution is

effective to determine MIC because it can test large number of antibiotics within a short period of time.[19] The same procedure until the adjustment of bacterial concentration to 0.5 McFarland standard was followed as previously mentioned in the Kirby-Bauer disc diffusion assay. The bacterial solution was further diluted 100 times. The experiment was such designed that each well would contain 100 µl of bacteria, 90 µl of MHB and 10 µl of test compounds with desired concentration. Negative control was prepared by adding MHB and vehicle only. An aliquot (10 µl) 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 was 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. Resazurin dye is used to turn from blue to orange if there is any viable cell. Following incubation, the MIC was visually determined as the lowest concentration at which colour development was inhibited.

Efflux pump activity study. Ethidium bromide-Agar Cartwheel method [20] was used to study whether the resistant clinical isolates have enhanced efflux pump activity or not. The bacterial solutions were adjusted to 0.5 McFarland Standard (KB test). Ethidium bromide (Et-Br) stock solution of concentration 100µg/ml was prepared and sterilized by filtration using a syringe filter (0.22µm pore size). Trypticase soy agar (TSA) plates with EtBr concentrations of 0.5, 1.0, 1.5, 2.0, and 2.5 µg/ml were prepared. 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 comparative control for fluorescence analysis. After inoculating the TSA plates, these were incubated, and were observed at 365 nm wavelength in a UV transilluminator. The minimum concentration of EtBr that produces fluorescence of the bacterial mass was recorded. Bacterial strains overexpressing EP show fluorescence in significantly higher concentrations than the reference strain. Efflux pump activity index is determined using the following formula:[21]

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.

EPI activity of vitexin 2''-*O*-xyloside and reserpine. The increased MIC of antibiotics can be attributed to the enhanced EP activities of the clinical isolates that were identified in the Et-Br agar cartwheel experiment. If the combination of a compound with an antibiotic reduces the MIC of that resistant antibiotic, then it can be assumed that the compound possesses efflux pump inhibitory activity. To study if vitexin 2''-*O*-xyloside can be an EPI, 128 µg/ml vitexin 2''-*O*-xyloside stock solution was prepared using 10% v/v ethanol-water as the solvent and reserpine was used as standard (32 µg/ml). Briefly, 100 µl of bacterial solution with McFarland standard 0.5 was added to each well. Then, 10 µl of antibiotic solution was added to each well and also reduced the concentration of the antibiotic to half to each well. Additional 10 µl test EPI solution of vitexin 2''-*O*-xyloside and reserpine was added to respective wells to observe their activity. The plate was incubated at 37°C for 18-24 h and the microplates are then interpreted in the UV microplate reader at 595 nm against blank. 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).[22]

Molecular docking studies. Molecular docking was performed to understand how the test compound may exert its action. The main objective of molecular docking is to get a ligand-receptor complex with optimized conformation and to possess less binding free energy. A three-dimensional (3D) homology model of NorA was developed using Modeller software (version 9.25) with UCSF Chimera. For this purpose, sequence of NorA (procured from UniprotKB, ID: P0A0J4) was subjected to BLASTp analysis against the PDB database to identify a suitable template. Ultimately YajR transporter from *Escherichia coli* (PDB ID: 3WDO) was selected based on the BLAST score, E-value and percent identity.[23] This template was used to build the homology model and subsequently the model was subjected to energy minimization using Modeller (version 9.25). The quality of the homology model was checked using PROCHECK (https://servicesn.mbi.ucla.edu/PROCHECK),[24] verify 3D (https://servicesn.mbi.ucla.edu/Verify3D)[25] and ERRAT (https://servicesn.mbi.ucla.edu/ERRAT)[26]. Molecular docking was carried out using Autodock Vina with PyRx. The structures of the selected ligands (reserpine, vitexin and vitexin 2"-*O*-xyloside) were optimized and energy minimized with density functional theory (DFT) model in Gaussian 09 (Revision D.01). The optimized structures were docked against the refined homology model of NorA with an exhaustiveness of 8 and maximization of search space. The best results were saved and analyzed with Discovery Studio Visualizer.

RESULTS AND DISCUSSION

Antimicrobial resistance in the clinical isolates of *S. aureus* is a global phenomenon. Apart from the MRSA, which is getting much attention due to their superbag characteristics, counterpart such as methicillin-susceptible *S. aureus* is also getting concern due to their wide variety of nosocomial infections. Sometimes it requires last-resource of antibiotic to treat such infections.[27] Among the different mechanisms, multidrug efflux pumps played a significant role in developing resistance towards several class of antibiotics. So, it is evident that the activity of efflux pumps can be reduced with adjuvant therapies which are called potential efflux pump inhibitors.[28] Due to the tedious process of the development of novel class of antibiotics, continuous efforts has been given to restore the efficacy of existing antibiotics. Several substrates such as reserpine, piperine, berberine, phenothiazine derivatives, omeprazole analogues etc has been searched for their EPI activity which can reverse the MIC breakpoints by inhibiting the activity of NorA MDR efflux pumps in *S. aureus*.[29,30] Present study was designed to evaluate the EPI activity of vitexin 2"-*O*-xyloside in clinical isolates of *S. aureus*.

The data recorded from the Kirby-Bauer disc diffusion assay gives an idea of the resistance pattern of the clinical isolates against the antibiotics, represented in Table 1. Most of the selected *S. aureus* strains showed a wide range of resistance pattern against all the tested antibiotics except chloramphenicol. All the isolates showed complete resistance against cefixime, ciprofloxacin and tetracycline. A systematic review on the antibiotic resistance pattern in Bangldesh showed that *S. aureus* exhibited high resistance to penicillin (89.7%), ampicillin (83.3%), ciprofloxacin (51.7%), co-trimoxazole (43.2%), tetracycline (43.5%) and amoxicillin (64.3%).[31] Same study reported that 6.8% sample was vancomycin resistant and 7 % against imipenem. In an another study, out of 44 stranis, 15

Table 1. Antibiotic sensitivity pattern of clinical isolates of *Staphylococcus aureus.*

Antibiotic disc	Resistant Isolates (%)	MIC value (µg/ml)
Tetracycline	100%	8 - 256
Chloramphenicol	0.00%	2 - 8
Linezolid	26.67%	4 - 64
Ciprofloxacin	100%	128 - 256
Amikacin	66.67%	4 - 128
Cefixime	100%	> 256
Azithromycin	20.00%	2 - 32

MRSA strains were detected and they were highly resistant to ciprofloxacin (93.33%), ceftriaxone (86.63%), azithromycin (73.33%), gentamycin (73.33%), and amoxiclav (66.67%).[32] Although all (100%) MRSA strains were sensitive to linezolid and 86.67% were sensitive to vancomycin, 26.67% isolates were found resistant in our study.

MIC test was performed to determine the lowest concentration of antibiotics required to inhibit the growth of the bacteria which is represented in Table-1. The MIC breakpoints for each antibiotics are evaluated based on the recommendation chart of BSAC, UK.[33] MIC values of of tetracycline, ciprofloxacin and cefixime were found in a wide range between 8-256, 128-256 and >256 µg/ml, respectively which indicated these clinical isolates are completely resistant to these antibiotics. It was found earlier that MIC breakpoints of cloxacillin was found >128 µg/ml for at least 3 strains of MRSA.[34]

Presence of efflux pump activity in the clinical isolates were evaluated by using ethidium bromide-agar cartwheel method and the results were published as efflux pump activity index in Table 2. Bacteria that fluoresced at the concentrations of EtBr higher than the reference standard are generally considered as efflux pump active isolates. Among the 15 isolates, 3 isolates were found to possess efflux pump in their structure and the MC_{EtBr} values were found between 1.5 to 2 µg/ml. In a recent study, it has been reported that single efflux pump NorA activity potentiates the evolution of antibiotic resistance against ciprofloxacin across the *S. aureus* isolates.[35] In another study from Iran, among the 34 clinical isolates of MRSA, *norA* gene was found to be present in 12 isolates.[36]

Table 2. Efflux pump activity of clinical isolates of *Staphylococcus aureus.*

Identifier	MC_{EtBr} (µg/ml)	Index
SA ATCC25923*	0.5	0
SA43158115P	2.0	3.0
SA21712133P	1.5	2.0
SA14240155P	1.5	2.0

* Reference standard

The ability of vitexin 2''-*O*-xyloside to reduce the required MIC value of tetracycline and ciprofloxacin in the efflux pump active clinical isolates of *S. aureus* was evaluated by broth microdilution technique. Σ FIC values were found in the range of 0.625 to 0.75 in efflux pump active isolates, indicating that vitexin 2''-*O*-xyloside at a concentration of 128 µg/ml showed additive effect when co-administered with tetracycline (Table 3). Reserpine, the positive standard showed indifferent effect due to observed Σ FIC values were in a range of 0.625 to 1.25 when co-treated with tetracycline in the same isolates. Coadministration of vitexin 2''-*O*-xyloside with ciprofloxacin gave indifferent effect in the same isolates. However, the MIC value of ciprofloxacin was reduced by 4-folds in those isolates whereas reserpine reduced the MIC values by 2-folds. It is evident that vitexin 2''-*O*-xyloside showed promising result in reducing the MIC values. Flavonoids generally showed efflux pump inhibitory activity in different clinical isolates of *S. aureus*. Two flavonoids from *Alpinia calcarata*, galangin and kaempferol showed ≥ 32-fold modulation in MIC values of ethidium bromide and norfloxacin in *S. aureus* strain.[37]

YajR transporter from *E. coli* was selected as the template for the homology model. This template had a resolution of 3.15 Å, R-Value (Work) 0.271 and R-Value (Free) 0.290. To produce a stable model, hydrogen were added to the model and the energy was minimized. After the model returned by Modeller; it was further refined and the quality of the model was assessed by using PROCHECK. Ramachandran plot showed that 92.5% of the residues were in favorable region, 9.3% were in allowed region, 1.2% were in generously allowed region with 1 residue in disallowed region. The environment profile of the model obtained using Verify 3D-1D was found to be mostly above zero (64.69% scored ≥0.2). ERRAT showed an overall quality factor of 91.644. The result of the molecular docking study is summarized in the Table 4. The results shows that both vitexin and vitexin 2''-*O*-xyloside had strong binding affinity to NorA protein, even when compared to a known inhibitor of NorA

such as reserpine. Reserpine and vitexin binds to the NorA homology model at similar site. However, reserpine interacts with the receptor mainly via hydrophobic interactions such as arene-arene interaction, arene-alkyl interaction and Van der Waals interaction whereas vitexin has several hydrogen bond interactions (with Asn137, Ser215 and Asp239) in addition to the hydrophobic interactions (Figure 1). The binding site of vitexin 2"-*O*-xyloside is different from that of reserpine and vitexin, and shared one residue (Phe216) with the binding site of reserpine.

Table 3. Effect of vitexin-2"-*O*-xyloside on the MICs of existing antibiotics.

Clinical isolates	MIC of antibiotic (µg/mL)	MIC of Vitexin-2"-*O*-xyloside (µg/ml)	MIC of antibiotic + Vitexin-2"-*O*-xyloside (128 µg/ml) (µg/mL)	Σ FIC value	MIC of reserpine (µg/mL)	MIC of reserpine (32 µg/ml) & antibiotic (µg/ml)	Σ FIC value
Tetracycline							
SA43158115P	128	512	64	0.625	32	16	0.625
SA21712133P	128	256	64	0.75	64	16	0.375
SA14240155P	256	512	128	0.75	64	64	1.25
Ciprofloxacin							
SA43158115P	256	256	128	1.0	32	32	1.125
SA21712133P	128	256	64	0.75	64	32	0.75
SA14240155P	128	512	128	1.25	64	64	1.5

Table 4. Binding affinity and binding interactions of the ligands with the NorA homology model.

Ligand	Binding affinity (kcal/mol)	Interacting Residues
Reserpine	-8.9	Phe140, Phe216, Phe221, Tyr225, Ala243
Vitexin	-9.5	Phe140, Asn137, Ser215, Asp239, Phe221, Ile240, Ala243
Vitexin 2"-*O*-xyloside	-8.3	Pro27, Val44, Phe216, Asn340, Pro344

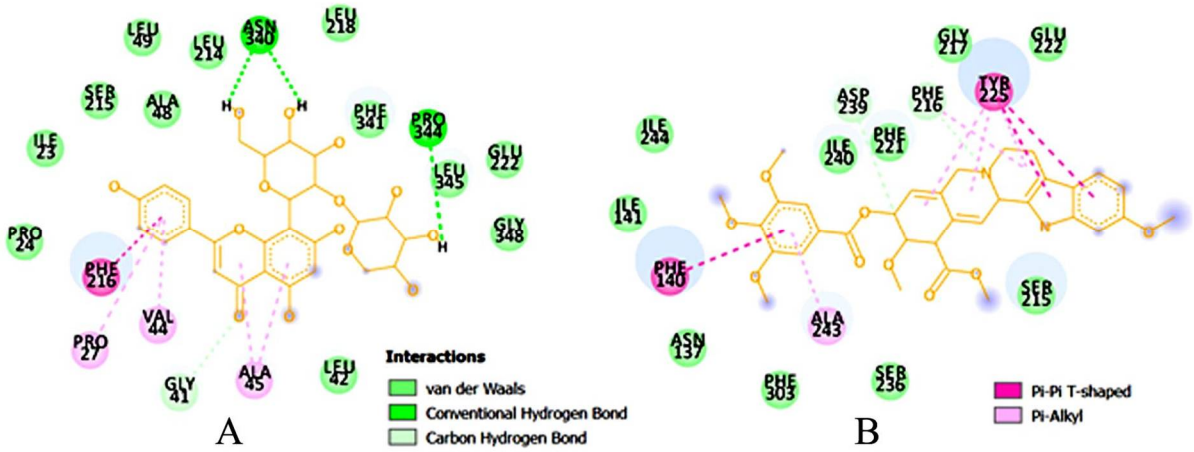

Figure 1. Interaction between vitexin-2"-*O*-xyloside, reserpine and NorA homology model. Figure A. Interaction with vitexin-2"-*O*-xyloside. Figure B. Interaction with reserpine

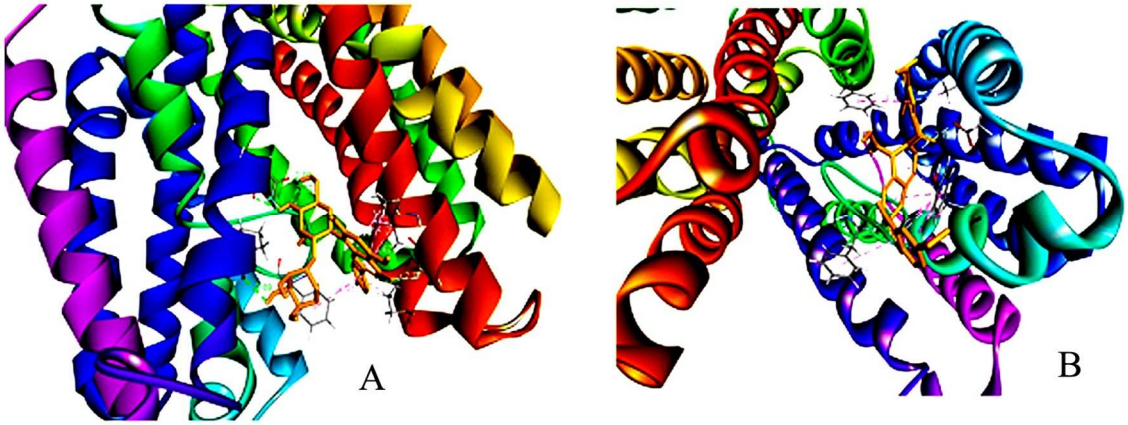

Figure 2. Binding sites of vitexin-2"-*O*-xyloside and reserpine with NorA homology model. Figure A. 3D view of vitexin-2"-*O*-xyloside. Figure B. 3D view of reserpine

Various approaches have been taken to produce homology models of NorA for the purpose of performing molecular docking studies. Different templates such as EmrD[38] and glycerol-3-phosphate transporter[39,40] from *E. coli* have been used to produce homology models. In this study, the model was constructed from YajR transporter after template search using SWISS-Model, Phyre2 and BLASTp analysis. Reserpine is a well known inhibitor of the NorA efflux pump in *S. aureus.*[38-41] Therefore, reserpine was used as a control in this study. This study found that Phe140 and Ala243 are important for the binding of both reserpine and vitexin to the NorA protein (Figure 2). These two residues provide hydrophobic surface for interaction. Vitexin also forms hydrogen bonds, strengthening its binding to the pocket compared to reserpine. Vitexin 2"-*O*-xyloside bound to a different site of the homology model, and Phe216 appeared to be an important residue for interaction for this compound. Previously, Phe140 and Phe216 were found to be a vital residue for binding.[39]

CONCLUSION

The alarmingly increased occurrence of multidrug resistance and the lack of newly appeared antibiotics in the market made it obvious to find an alternative method of making the existing resistant antibiotics effective again. This study was an effort toward achieving that goal. The MIC results showed that vitexin 2"-*O*-xyloside may have the potential to inhibit the efflux pump of specific isolates of *S. aureus.* This was further reinforced by the molecular docking results that show a good binding interaction between vitexin 2"-*O*-xyloside and the receptor binding site. Besides the result was compared to that of reserpine, a well-known EPI. The question of whether it can be considered as a potential EPI is critical, and requires further studies.

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Conflict of Interest. The authors declare no conflict of interest.

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