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ORIGINAL ARTICLE

Biological Evaluation and Molecular Docking Study of Metabolites from *Salvadora persica* L. Growing in Egypt

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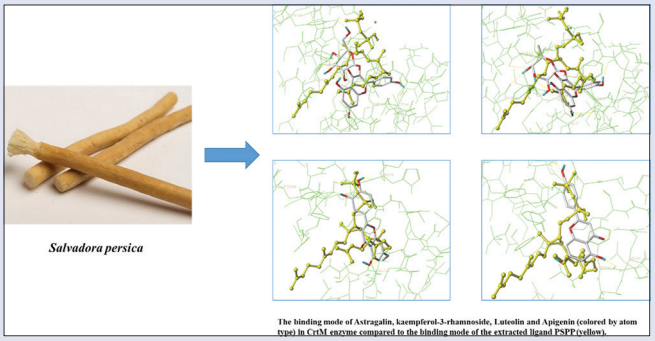
ABSTRACT

Background: Methicillin-resistant *Staphylococcus aureus* (MRSA) is a resistant staph bacterium to several antibiotics causing several lives-threating diseases such as pneumonia and sepsis. Meswak, *Salvadora persica*, exhibited promising antimicrobial properties before. **Objective:** Exploring the anti-MRSA activity of *S. persica* L. metabolites and its mechanism of action on a molecular level. **Materials and Methods:** Structure elucidation of the isolated metabolites was carried out by spectroscopic data (one-dimensional and two-dimensional nuclear magnetic resonance). The biological activities of the isolated metabolites against MRSA were evaluated and the molecular mode of action against the dehydrosqualene synthase enzyme have been done. **Results:** Four compounds have been isolated and identifies to be; apigenin (1), luteolin (2), astragalin (3), and kaempferol-3-O-rhamnoside (4). Compounds 1–4 showed good anti-MRSA activities with IC_{50} values of 10.3, 11.5, 3.5, and 4.5 µg/mL, respectively. In consistent, astragalin and kaempferol-3 rhamnoside showed close high docking scores. Herein, we are reporting the molecular determinates of activity of these new scaffolds as anti-MRSA, which would be of great importance to developing new anti-MRSA candidates.

Key words: Anti-methicillin-resistant *Staphylococcus aureus*, antimicrobial activities, dehydrosqualene synthase enzyme, docking molecular study, *Salvadora persica*

SUMMARY

- A phytochemical study of *Salvadora persica* ethanolic extract led to the isolation and identification of four compounds including the following: apigenin (1), luteolin (2), astragalin (3), and kaempferol-3-O-rhamnoside (4). Compounds 1–4 showed good anti-methicillin-resistant *Staphylococcus aureus* (MRSA) activities with IC_{50} values of 10.3, 11.5, 3.5, and 4.5 µg/mL, respectively. The molecular mode of the action of isolated compounds against the dehydrosqualene synthase enzyme has been explored. Binding modes studies revealed that astragalin and kaempferol-3 rhamnoside are highly expected to be potent dehydrosqualene synthase inhibitors with close high docking scores and close high anti-MRSA activities. Be a poor inhibitor, while apigenin did not exhibit a proper binding mode to exert any inhibitory activity.



The binding mode of Astragalin, kaempferol-3-rhamnoside, Luteolin and Apigenin (colored by atom type) in CrtM enzyme compared to the binding mode of the extracted ligand PSPP (yellow).

Abbreviations Used: 1D: One-dimensional; 2D: Two-dimensional; CC: Column chromatography; COSY: Correlations spectroscopy; DMSO: Dimethyl sulfoxide; HMBC: Heteronuclear multiple-bond correlation experiment; HRESIMS: High-resolution electrospray ionization mass spectrometry; HSQC: Heteronuclear single-quantum correlation; IR: Infrared; MRSA: Methicillin-resistant *Staphylococcus aureus*; NMR: Nuclear magnetic resonance; RP: Reversed phase; TLC: Thin-layer chromatography; UV: Ultraviolet; VLC: Vacuum liquid chromatography.

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INTRODUCTION

Natural products are unlimited sources for anti-infectives, either from endophytic fungi,[1,2] marine natural products,[3] or plants.[4,5] *Salvadora persica* L. of the family *Salvadoraceae* is reported to have a potent activity for dental complaints.[6] *S. persica* is one of the most commonly used medicinal plants for teeth cleaning and oral hygiene among the global Muslim community.[6] A variety of chemical components have been isolated from *S. persica* extracts. Some of these biologically active chemical constituents as sodium chloride, potassium chloride, salvadourea, alkaloids, and oleic and linoleic acids have been suggested to contribute the cleansing efficacy of meswak by leaching out in saliva and for their antifungal properties.[7,8]

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a prominent example of antimicrobial resistance. For half a century, MRSA strains demonstrated an amazing ability to develop antimicrobial resistance to many antimicrobial agents with versatile extent and ranges.[9] Prompted by these facts, there is

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an urgent need to adopt new therapeutic strategies for the development and discovery of new effective antimicrobial agents against MRSA.

Literature survey revealed a series of alternative therapeutic strategies, which are mainly based on targeting microbial virulence rather than survival.[10] One of these strategies involves the neutralization of bacterial virulence factor to increase the susceptibility of pathogenic bacteria to immune system clearance.[11]

In *S. aureus,* a virulence factor staphyloxanthin (STX) is produced, which is a carotenoid pigment that acts as an antioxidant for protection against host immune system-generated reactive oxygen species. Inhibition of STX biosynthesis makes *S. aureus* an easy target for the host immune system.[11-14] A metabolic pathway, which involves dehydrosqualene synthase enzyme, is responsible for the synthesis of STX . In this pathway, two molecules of Farnesyl PyroPhosphate (FPP) are condensed by the enzyme dehydrosqualene synthase (CrtM), forming one molecule of presqualene diphosphate (PSPP), which is then converted by the same enzyme to dehydrosqualene, which is further transformed to STX.[15]

The dehydrosqualene enzyme contains two binding sites S1, S2 and a cluster of 3 Mg^{+2} ions above S1 known as (PPi) binding site, which is responsible for the removal of diphosphate groups and ionization of FPP and PSPP molecules. The S1-binding site is highly bent, and although it seems shorter, it is actually longer whereas the S2-binding site is quite straight. The two binding sites S_1 and S_2 are occupied by two FPP molecules.[15]

The overall enzymatic reaction in dehydrosqualene synthase enzyme involves the ionization of one FPP molecule at S1-binding site to form 1' farnesyl carbocation, which then undergoes nucleophilic attack by C2,3 double bond in the second FPP molecule at S2-binding site to give PSPP after proton loss. At this step, PSPP is a condensation product of the two FPP molecules and occupies both binding sites S1 and S2. PSPP is further ionized by the removal of its diphosphate group to give cyclopropyl carbocation derivative, which undergoes rearrangement to give dehydrosqualene.[15]

Dehydrosqualene synthase inhibitors may act as cationic isosteres for farnesyl carbocation at S1-binding site or as transition state-reactive intermediate analogs for cyclopropyl carbocation species of PSPP at S2-binding site.[15]

Lin *et al.*[15] rationalized the activity of a series of potent dehydrosqualene synthase (CrtM) inhibitors based on their structure and binding mode in the target site. It is concluded that the basic structure features for potent inhibitors are represented by the presence of a straight aromatic backbone with a side-chain bulky hydrophobic ring and aliphatic side chain ended with phosphosulfonate group. The straight aromatic backbone occupies the linear S2-binding site, while the side-chain bulky ring readily fit into the lower pocket of the bent S1-binding site, where binding affinity is mainly dominated by hydrophobic interaction. The phosphosulfonate group moves to the PPi-binding site and bind through chelation with $(Mg^{+2})_3$ cluster.

Many flavone natural products have been reported for their anti-MRSA activity.[10,16-18] Flavone derivatives, apigenin and luteolin, were found responsible for inhibiting the production of virulence factors alpha-hemolysin and alpha-toxin in *S. aureus*, respectively.[19,20] Recently, they were reported for increased susceptibility of MRSA strains to β-lactam antibiotics.[21,22] Lee *et al.* reported the ability of luteolin to inhibit STX production.[23] Other flavone derivatives, astragalin and kaempferol-3-O-rhamnoside, are found to have structural similarity with the basic structural requirements for potent STX inhibitors due to having a straight aromatic backbone and the side chain bulky sugar ring. Based on these findings, compounds are expected to act as dehydrosqualene synthase inhibitors. All the four compounds have activity against MRSA with variable extent.

A molecular modeling approach and a docking study were carried out for exploring the possible binding mode of these compounds in dehydrosqualene synthase enzyme in correlation to their anti-MRSA activity.

MATERIALS AND METHODS

General experimental procedures

Ultraviolet spectra were determined with Pye Unicam spp. 1750 spectrophotometer. EIMS was carried on Scan EIMS-TIC, VG-ZAB-HF, and X-mass (158.64, 800.00) mass spectrometer (VG Analytical, Inc.). The 1H- and 13C nuclear magnetic resonance (NMR) measurements were obtained with a Bruker Avance III (400) NMR spectrometer operating at 400 MHz (for 1H) and 100 MHz (for 13C) in CD3OD or dimethyl sulfoxide-d6 solution, and chemical shifts were expressed in δ (ppm) with reference to Teteamethylsilane (TMS), and coupling constant (J) in Hertz. 13C multiplicities were determined by the DEPT pulse sequence (135o). Si gel (Si gel 60, Merck) and Sephadex LH-20 (Pharmacia) were used for open column chromatography. Solid phase extraction was performed on SPE-C18 cartridges (A Strata column, Phenomenex, USA). Thin-layer chromatography was carried out on precoated silica gel 60 F254 (Merck) plates. Developed chromatograms were visualized by spraying with 1% vanillin-H_2SO_4, followed by heating at 100°C for 5 min, or spraying with ammonia or aluminum chloride solutions.

Plant material

S. persica L. was collected from Sinai desert, Egypt, on Jun 2016. A voucher specimen (SP1) has been deposited in the Pharmacognosy Department, Faculty of Pharmacy, Al-Azhar University, Cairo, Egypt.

Extraction and isolation

Air-dried powdered aerial parts of *S. persica L.* (2 kg) were exhaustively extracted by maceration with 70% C_2H_5OH (7 L × 3) at room temperature. The combined ethanolic extracts were concentrated under vacuum at 60°C to dryness. The concentrated ethanolic extract (450 g) was suspended in distilled water (600 ml) and partitioned successively with n-hexane, ethyl acetate, and n-butanol to give 15 g, 15 g, and 20 g, respectively. The ethyl acetate extract was subjected to a silica gel column eluted with n-hexane: ethyl acetate 95:5–10:90 to obtain six fractions of A (500 mg), B (700 mg), C (450 mg), D (850 mg), E (900 g), and F (800 mg). Fraction B was rechromatographed on silica gel CC eluted with n-hexane: ethyl acetate 90:10–80:20 to give compound 1 (45 mg). Fraction D was chromatographed on a Sephadex LH-20 column eluted with CH_2Cl_2: CH_3OH 50:50 to give compound 3 (20 mg) and subfraction D-1 (90 mg). The subfraction D-1 was further subjected to solid-phase extraction (RP-C18) using 50:50-60:40 CH_3OH: H_2O system to obtain compound 2 (25 mg). Fraction E was subjected to Sephadex LH-20 columns eluted with CH_3OH to afford compounds 4 (50 mg).

Antimicrobial activity

The antimicrobial screen tests were done in the National Research Center, Dokki, Cairo, Egypt, for compounds 1–4 for their ability to inhibit a panel of five bacteria and five fungi those are pathogenic to humans for antimicrobial activity against *S. aureus*, MRSA, *Escherichia coli*, *Pseudomonas aeruginosa*, *Mycobacterium intracellulare*, *Candida albicans, Candida glabrata*, *Candida krusei*, *Cryptococcus neoformans*, and *Aspergillus fumigatus*, as the following procedure.[24-27]

Primary screen: Crude extracts are initially tested at 50 μg/mL in duplicate and percent inhibitions (% inh.) are calculated relative to negative and positive controls. Extracts showing ≥50% inhibition proceed to the secondary assay.

Secondary assay: In the secondary assay, samples dissolved to 20 mg/mL (crude extracts and some column fractions) are tested at 200, 40, 8 µg/mL, and IC_{50}s versus all 10 microbial strains are reported. Samples dissolved to 2 mg/mL (pure compounds and some column fractions) are tested at 20, 4, 0.8 µg/mL, and IC_{50}s versus all 10 microbial strains are reported. Pure compounds that have an IC_{50} of ≤7 µg/mL in the secondary OI assay proceed to the tertiary assay.

Tertiary assay: In the tertiary assay, pure compounds are tested versus all 10 microbes at 20, 10, 5.0,… 0.02 µg/mL and IC_{50}s are calculated. In addition to the IC_{50}, the minimum inhibitory concentration (MIC) and either the minimum fungicidal concentration (MFC) or minimum bactericidal concentration (MBC) are reported.

The MIC is the lowest test concentration (in µg/mL) that inhibits the organism 100%. The MFC or MBC is the lowest test concentration (in µg/mL) that kills the organism. While a pure compound may have an MIC, the cells may still be alive, just not growing. The MFC and MBC is a way to monitor the "cidality" or killing the ability of the test sample. All IC_{50}s are calculated using the XLF, it fits curve fitting software. The antifungal drug control is amphotericin B, and the antibacterial drug control is ciprofloxacin.

Molecular modeling and docking study

A docking study was done using the X-ray crystal structure of *S. aureus* dehydrosqualene synthase enzyme bound to PSPP from Lin *et al.*[15] The crystal structure was obtained from Protein Data Bank (http://www.rcsb.org) with the code 3 LGZ at a resolution 2.41 A°. Modules of Program Sybyl X 2.1.1 (Tripos Inc., St. Louis, MO) were used for the preparation of protein, ligands, and carrying out the docking study.

Using structure preparation tool from Sybyl for the preparation of protein, bonds with metal were deleted, charges were added to termini, hydrogens were added with H-bonding orientation, and side chain amides were fixed. ProTable module was used for checking conformational problems in the protein structure. Backbone torsion angles, PHI, and PSI were checked using Ramachandran plot.[28,29] Location of buried nonpolar residues/exposed polar residues was examined. Energy minimize computational tool was used for energy refinement of protein structure. MMFF94 charges[30] were added to the ligand-protein complex. The model was then subjected to energy minimization with the following adjusted parameters (Method: Powell, Initial Optimization: None; Termination: Gradient at 0.5 kcal/mol; Max Iterations: 5000; dielectric Function: Constant; dielectric constant: 1; and Rest of Parameters: SYBYL-X Default).

The test compounds were optimized using Prepare Ligand application from Sybyl as a Quick 3D Job. The generated 3D structures were first checked and corrected for axial and equatorial conformation of the substituents on sugar ring, then further optimization was done through energy minimization with the following adjusted parameters (Method: Powell, Initial Optimization: None; Termination: Gradient at 0.005 kcal/mol; Max Iterations: 5000; Force Filed: MMFF94s; charges: MMFF94; dielectric Function: Constant; Dielectric Constant: Constant).

Docking experiment

The tested natural flavone derivatives in this study were docked to dehydrosqualene synthase enzyme (CrtM) using the Surflex-Dock in Sybyl and the most accurate docking mode GeomX. This software uses an empirical scoring function and a patented search engine to dock ligands into a protein's binding site.[31] In Surflex-Dock, docking is guided by the protocol, an idealized representation of a ligand that makes every potential interaction with the binding site. Surflex-Dock is successful at eliminating false positive, while still retaining a large number of active compounds.

In this module, the ligand (PSPP) was first extracted from protein and water was removed. The ProtoMol was generated guided by the ligand with the threshold and bloat parameters set at 0.5 and 0, respectively. In Surflex-Dock options, the flexibility of protein was considered by allowing the movement of hydrogens in the binding site. Surflex-Dock uses an empirically derived scoring function that is based on the binding affinities of protein–ligand complexes and their X-ray structures.[32] The validity of the model was checked by including the extracted ligand with the tested compounds in the docking run.

RESULTS AND DISCUSSION

Dried aerial parts of *S. persica L.* were exhaustively extracted with ethanol after defatting of the powdered plant with *n*-hexane. The obtained crude extract was then treated by combined chromatographic separations. Four compounds were isolated [Figure 1]. Their structures were elucidated using different spectroscopic techniques. The isolated metabolites were identified as follows: apigenin[33] (1), luteolin[34] (2), astragalin[35] (3), and kaempferol-3-O-rhamnoside[36] (4). The isolated compounds were evaluated for their antimicrobial activity. The examined compounds did not show promising activities against microbes, except for MRSA. Compounds 1–4 showed activities against MRSA with IC_{50} values of 10.3, 11.5, 3.5, and 4.5 µg/mL, respectively. We are suggesting that these compounds are interfering with dehydrosqualene synthase enzyme.

To explore the binding mode of the tested flavone derivatives in the target enzyme (CrtM) compared to the binding modes of the reported potent dehydrosqualene synthase inhibitors, a molecular modeling approach and a docking study were carried out. The crystal structure of *S. aureus* CrtM-PSPP complex (3 LGZ. pdb) at a resolution of 2.041A° was optimized and minimized as shown in Figure 2 with PSPP ligand in ball and stick form. It showed that the cyclopropane central nucleus of PSPP is attached to two aliphatic unsaturated side chains and a diphosphate group. One of the side chains occupies the highly bent S1 pocket, while the other occupies the straight S2 pocket. Binding affinity in these pockets is mainly due to hydrophobic interaction with surrounding residues such as (VAL 37, LEU 141, TYR41, and CYS44) in S1 pocket and (LEU160, ILE241, LEU141, and PHE22) in S2 pocket. The diphosphate group is protruding outside to occupy the PPi-binding site where binding affinity is dominated by hydrogen-bonding interaction with (ARG45, ARG171, ASN168, and TYR248) and chelation with MG290. Analysis of the chain backbone using Ramachandran plot showed a normal distribution of most points in the favored, allowed, and generously allowed regions with only one amino acid residue SER82 classified as an outlier [Figure 3]. Tested compounds were docked into the identified binding site using the Surflex-Dock module in Sybyl in combination

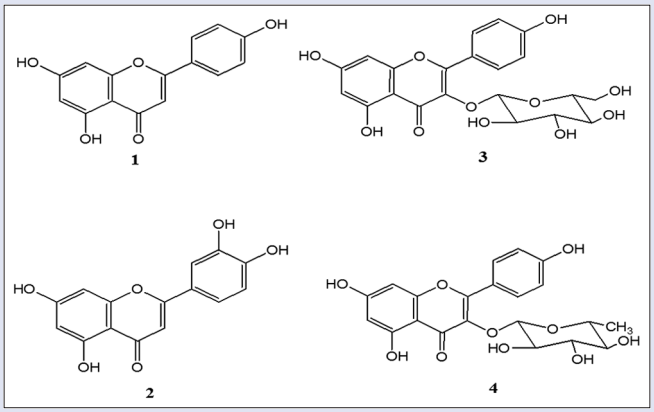

Figure 1: Compounds 1–4

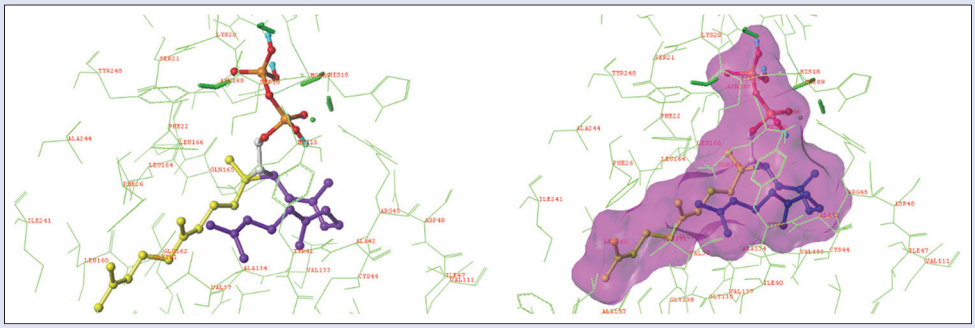

Figure 2: Binding site of *Staphylococcus aureus* CrtM dehydrosqualene synthase inhibitors showing the position of ligand presqualene diphosphate (ball and stick). The side chain in the bent S1 pocket (blue), side chain in the straight S2 pocket (yellow) and the diphosphate group colored by atom type

Table 1: Biological activity of flavone derivatives against methicillin-resistant *Staphylococcus aureus* strain and their docking scores against dehydrosqualene synthase enzyme

	MIC (μg/mL)	MIC (micromolar)	Docking score
Astragalin	3.5	7.8	10.87
Kaempferol-3-rhamnoside	4.5	10.4	9.48
Luteolin	11.5	38.11	6.64
Apigenin	10.3	40.17	5.58

MIC: Minimum inhibitory concentration

with a total score function for the prediction of binding affinity [Table 1]. The extracted ligand PSPP which was included in the tested compounds demonstrated the highest docking score, which confirms the validity of the model. The docking model demonstrated high accuracy in the prediction of the binding mode of PSPP compared to its actual binding mode from the crystal structure [Figure 4]. Compound astragalin demonstrated the highest docking score among the tested compounds which is in concordance with its being the most biologically active as anti-MRSA. Although astragalin showed structure similarity to the basic structural features of potent dehydrosqualene synthase inhibitors, the unexpected binding mode was exhibited [Figure 5]. The straight aromatic backbone occupies S1 pocket instead of S2 pocket, and the side sugar moiety occupies the upper part of S2 pocket most close to the diphosphate group of PSPP instead of the lower terminus of S1 pocket. The unexpected binding mode did not affect the docking score, as the compound demonstrated very good fitting in the binding site. In S1 pocket, affinity was dominated by hydrophobic interaction of the aromatic backbone with phenyl ring of TYR41 and hydrogen-bonding interaction of the phenolic (OH)s in the aromatic backbone with VAL37 and CYS44. The side sugar moiety showed very good fitting in S2-binding site through hydrogen bonding of OH substituents with GLY161 and similarity to diphosphate group in H-bonding to TYR248. Kaempferol 3-rhamnoside had close docking score to astragalin, close anti-MRSA activity, and close similarity in the binding mode at the binding site [Figure 6]. Luteolin demonstrated poor fitting in the binding site with low-docking score. Only small part of the aromatic backbone occupies the upper terminus of S1 pocket with the majority of it protruding outside with similar spatial orientation to the diphosphate group [Figure 7]. Poorer fitting in the binding site was demonstrated by apigenin which is expressed in much lower docking score and much lower anti-MRSA activity. Although the aromatic backbone failed to occupy S1 or S2 pocket, it showed much better similarity in the spatial orientation to the diphosphate group [Figure 8]. There is a very close relationship between the docking scores and the reported anti-MRSA activity. Astragalin and kaempferol-3rhamnoside demonstrated close high-docking scores and close high anti-MRSA activity. After studying the binding modes of the tested compounds, it seems that astragalin and kaempferol-3 rhamnoside are highly expected to be potent dehydrosqualene synthase inhibitors.

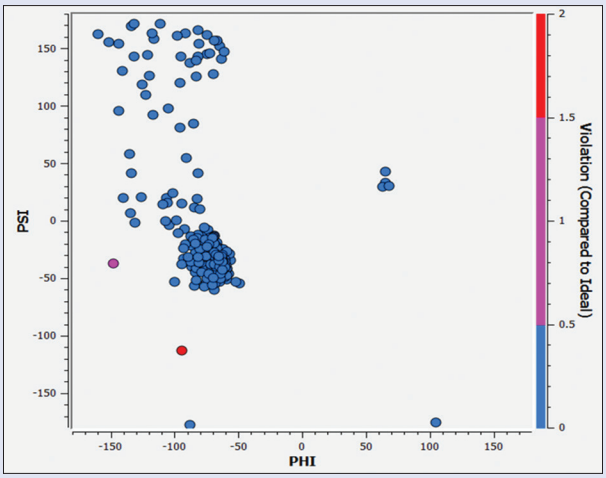

Figure 3: Ramachandran plot of the protein crystal structure 3LGZ (blue-favored), (magenta-allowed) and (red-not allowed)

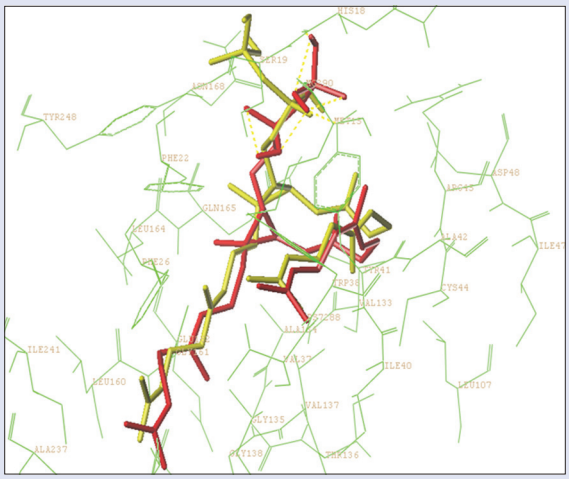

Figure 4: The predicted binding mode of presqualene diphosphate (red) compared to the reported binding mode of presqualene diphosphate (yellow) from the crystal structure showing high degree of accuracy

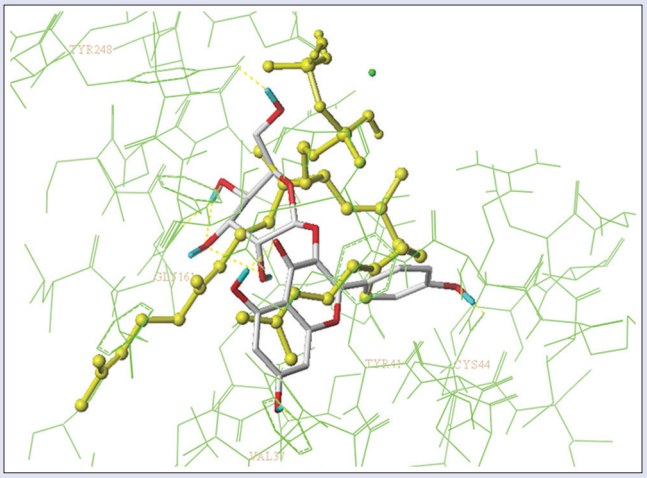

Figure 5: The binding mode of astragalin (colored by atom type) in CrtM enzyme compared to the binding mode of the extracted ligand presqualene diphosphate (yellow)

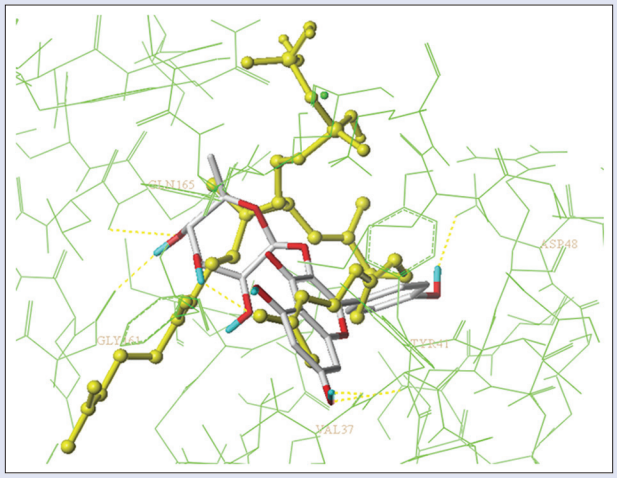

Figure 6: The binding mode of kaempferol-3-rhamnoside (colored by atom type) in CrtM enzyme compared to the binding mode of the extracted ligand presqualene diphosphate (yellow)

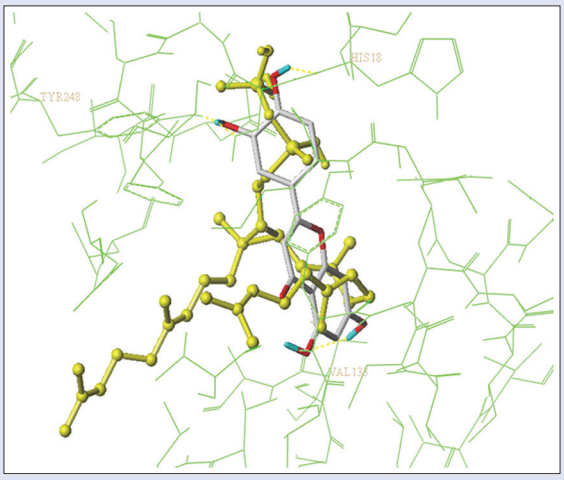

Figure 7: The binding mode of luteolin (colored by atom type) in CrtM enzyme compared to the binding mode of the extracted ligand presqualene diphosphate (yellow)

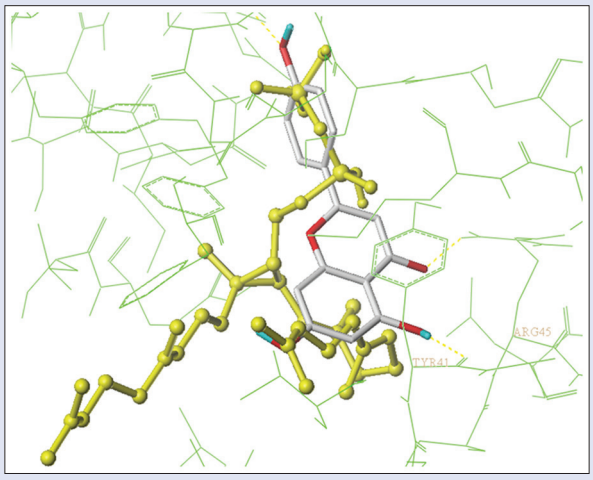

Figure 8: The binding mode of apigenin (colored by atom type) in CrtM enzyme compared to the binding mode of the extracted ligand presqualene diphosphate (yellow)

Luteolin is expected to be a poor inhibitor, while apigenin did not exhibit a proper binding mode to exert any inhibitory activity.

CONCLUSION

Four compounds have been Isolated from *S. persica* L. growing in Egypt and identified to be the following; apigenin (1), luteolin (2), astragalin (3), and kaempferol-3-O-rhamnoside (4). All compounds exhibited good anti-MRSA activities with IC_{50} values of 10.3, 11.5, 3.5, and 4.5 µg/mL, respectively. The molecular determinates of compounds activities have been expected to be against the dehydrosqualene synthase enzyme.

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Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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