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ORIGINAL ARTICLE

Evaluation and Understanding the Molecular Basis of the Antimethicillin-Resistant *Staphylococcus aureus* Activity of Secondary Metabolites Isolated from *Lamium amplexicaule*

Mohammed M. Ghoneim[1,2], Arafa Musa[1,3], Atef A. El-Hela[1], Khaled M. Elokely[4]

[1]Department of Pharmacognosy, Faculty of Pharmacy, Al-Azhar University, Cairo, Egypt, [4]Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Tanta University, Tanta, Egypt, [2]Department of Natural Products, School of Pharmacy, Almaarefa Colleges for Science and Technology, Riyadh, [3]Department of Pharmacognosy, College of Pharmacy, Jouf University, Sakaka, Al-Jouf, Saudi Arabia

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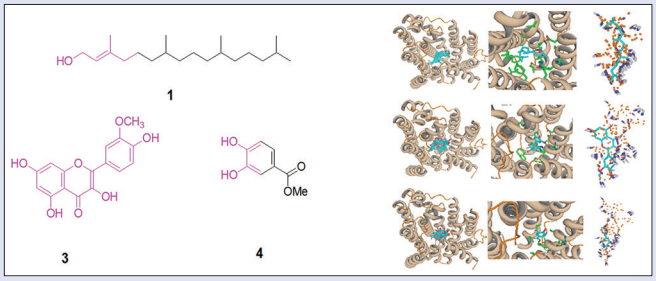
ABSTRACT

Background: The genus Lamium includes about forty annual or perennial plants distributed everywhere, it has significant biological activities including antimicrobial, antioxidant and antischistosomal effects. However, no detailed reports about the antimicrobial (Anti-MRSA) effect of the isolated metabolites. **Objective:** Studying the mechanism of action of the antimicrobial (Anti-MRSA) activity of the isolated metabolites. **Materials and Methods:** The EtOAc extract of *L. amplexicaule* was subjected to different chromatographic methods to isolate the secondary metabolites, and the isolated compounds were elucidated by spectroscopic techniques. The antimicrobial activity against strains of microorganisms was performed according to Minimum Inhibitory Concentration, the study of Anti-MRSA activity was explained by molecular docking against CrtM enzyme. **Results:** Phytochemical study of the aerial parts of L. amplexicaule resulted in the isolation of 5 known compounds; phytol (1), *β*-sitosterol (2), isorhamnetin (3), 3,4-dihydroxy-methyl benzoate (4), and hydroxynervonic acid (5). The antimicrobial activity of the isolated metabolites revealed that compounds 1, 3, and 4 have pronounced antimethicillin-resistant *Staphylococcus aureus* (MRSA) effect. **Conclusion:** These all known compounds were firstly isolated from *L. amplexicaule*. Three of them showed pronounced anti- MRSA effect, The mechanism of action against dehydrosqualene synthase enzyme was established. In addition, the study of molecular determinates of activity of these new scaffolds as anti-MRSA has a great importance for the development of new anti-MRSA candidates.

Key words: Anti-MRSA, CrtM, docking, *L. amplexicaule*

SUMMARY

- Five known compounds were isolated from Lamium amplexicaule, the biological screening of them was performed against -MRSA candidates and only three showed promising effect. The mechanism of active compounds was postulated through docking studies.



Abbreviations used: L. amplexicaule: Lamium amplexicaule; CrtM: Dehydrosqualene synthase; MRSA: Methicillin-resistant strains of S. aureus; STX: Staphyloxanthin; UV: Ultraviolet-visible; TLC: Thin-layer chromatography; ESI/MS: Electrospray mass spectrometry; VLC: Vacuum liquid chromatography; L: Liter

Correspondence:

Dr. Arafa Musa,
Department of Pharmacognosy,
Faculty of Pharmacy, Al-Azhar University,
Cairo 11371, Egypt.
E-mail: arafa_1998@yahoo.de
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INTRODUCTION

Staphylococcus aureus is the major cause of many hospital-acquired (nosocomial) or community-acquired human infections.[1] Many strains of *S. aureus* developed resistance to the currently used antimicrobial agents; however, the emergence of methicillin-resistant strains of *S. aureus* (MRSA) poses notable challenges to drug developers.[1] The need to find new anti-MRSA therapies is highly demanding. Based on the recent reports of the Centers for Disease Control and Prevention (CDC), the number of patients that are dying from invasive MRSA each year in the US is more than those from AIDS.[1,2] One of the approaches is through the inhibition of the enzyme dehydrosqualene synthase (diapophytoene synthase or CrtM) which is responsible for the synthesis of the bacterial virulence factors.[3] This strategy is aimed to potentiate the effect of classical antibiotics and reduce the development of drug resistance. CrtM, the virulence factor staphyloxanthin (STX), is a golden carotenoid pigment [Figure 1]. STX increases the resistance of *S. aureus* to innate immune clearance by deactivating the reactive oxygen species generated by neutrophils and macrophages.[4,5]

Lamiaceae (Labiatae) family is one of the wide spread families that contain numerous plant species with great medicinal uses. Many of them are very common in Mediterranean region.[6] The genus *Lamium* L. includes about forty annual or perennial herbaceous plants that distributed throughout Europe, Asia, and Africa.[7] *Lamium amplexicaule* has significant biological activities including antimicrobial,

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antioxidant,[8] and antischistosomal.[9] Monoterpene glycosides, flavonol glycosides, essential oils, iridoid glucosides, phytol, and β-sitosterol have been isolated from *L. amplexicaule*.[10-12] In this study, the isolation and antibacterial evaluation of secondary metabolites from *L. amplexicaule* were reported. The mechanism of binding affinity of these structures on CrtM as well as the putative flexibility of CrtM that is supposed to be of great benefit against MRSA was studied.

MATERIALS AND METHODS

General experimental procedures

Ultraviolet-visible (UV) spectra were determined with a Hitachi 340 spectrophotometer, Tokyo, Japan. Infrared spectra were carried out on a Nicolet 205 Fourier-transform infrared spectrometer connected to a Hewlett-Packard Color Pro Plotte, USA. Nuclear magnetic resonance spectra were recorded on a Varian Mercury (USA) 400 MHz spectrometer at 400 (^{1}H) and 100 MHz (^{13}C) in DMSO-*d*6 or CDCl3-*d* solution and chemical shifts were expressed in δ (ppm) with reference to TMS and coupling constant (*J*) in Hertz. ^{13}C multiplicities were determined by the distortionless enhancement by polarization transfer pulse sequence (135°). The electron ionization mass spectral spectra were measured using El/MS 502 mass spectrometer having a direct inlet system and operating at 70 eV. The ESI/MS spectra were measured using a Bruker BioApex-FTMS with electrospray ionization, USA. Column chromatographic separation was performed on silica gel 60 (Si gel 60 [0.063–0.200 mm] for column chromatography [70–230 mesh American standard test sieve series], Merck, Darmstadt, Germany) and Sephadex LH-20 (Pharmacia, Uppsala, Sweden). Thin-layer chromatography (TLC) was performed on precoated TLC plates with silica gel 60 F_{254} (0.2 mm, Merck, Darmstadt, Germany). Developed chromatograms were visualized first by UV-lamp (254 nm and 365 nm), Cole-Parmer, USA, followed by spraying with 1% vanillin-H_2SO_4 and heating at 100°C. All mobile phase solvents were purchased from Scharlau, Chemie S.A., Spain. The used glass columns were of different sizes and dimensions 120 cm × 7 cm, 90 cm × 5 cm, 70 cm × 3 cm, and 50 cm × 2 cm.

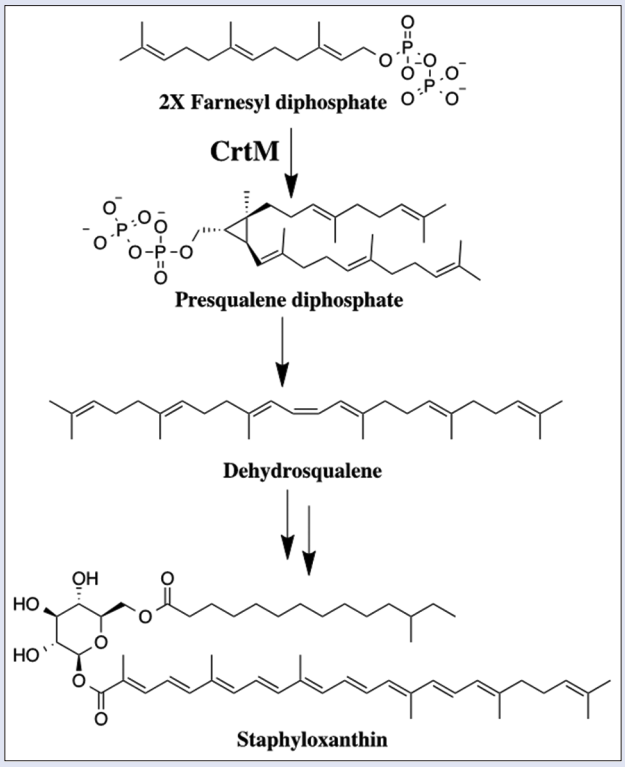

Figure 1: Biosynthesis of staphyloxanthin by CrtM

Plant material

L. amplexicaule aerial parts were collected from Orman Botanical Garden, Giza, Egypt. The plant was kindly identified and authenticated by Dr. Abdu Mareey, Professor of plant taxonomy, Faculty of Science, Al-Azhar University, Cairo, Egypt. A voucher specimen (LA-1) has been deposited in the Pharmacognosy Department, Faculty of Pharmacy, Al-Azhar University, Cairo, Egypt.

Extraction and isolation

The air-dried powdered plant material (1 kg) was exhaustively extracted by maceration with 70% EtOH (7 L × 3) at room temperature. The combined ethanolic extracts were evaporated under reduced pressure to afford 53 G dry residue. The residue was subjected to vacuum liquid chromatography (VLC) on silica gel (600 G) using 2.0 L each of petroleum ether, EtOAc, and MeOH, respectively. All three fractions were evaporated under reflux to yield 27 G (petroleum ether fraction), 13 G (EtOAc fraction), and 11 G (MeOH fraction). The EtOAc fraction (13 G) was subjected to a series of silica gel column chromatography, compound 1 was eluted by *n*-hexane-ethyl acetate mixture as mobile phase in a ratio of 90:10, and compound 2 was eluted by 85:15 ratio, while compound 3 was eluted by the same solvent mixture with ratio of 65:35. The petroleum ether fraction (27 G) was subjected to subsequent elution on silica gel column chromatography to yield compounds 4 and 5 using *n*-hexane-ethyl acetate mixture as mobile phase in ratios of (95:5) till (75:25), respectively. The isolated compounds were further purified on gel filtration using Sephadex LH-20 as stationary phase and methanol as mobile phase.

Antimicrobial activity

It was performed according to the protocol of Minimum Inhibitory Concentration, compounds 1–5 were tested for antimicrobial activity against *S. aureus* ATCC 29213, MRSA ATCC 33591 MRSA, *Escherichia coli* ATCC 35218, *Pseudomonas aeruginosa* ATCC 27853, *Mycobacterium intracellulare* ATCC 23068, *Candida albicans* ATCC 90028, *Candida glabrata* ATCC 90030, *Candida krusei* ATCC 6258, *Cryptococcus neoformans* ATCC 90113, and *Aspergillus fumigatus* ATCC 204305, ciprofloxacin, and amphotericin-B were used as positive controls for bacteria and fungi, respectively.[13-15] All organisms were obtained from the American Type Culture Collection (Manassas, VA). For all organisms, susceptibility testing was performed using a modified version of the Clinical and Laboratory Standards Institute (formerly NCCLS) methods and optical density was used to monitor growth [Table 1].

Table 1: The antimicrobial activity (minimum inhibitory concentration) of the isolated compounds

Microorganism	IC_{50} (μg/mL)			
	Compound 1	Compound 3	Compound 4	Ciprofloxacin
S. aureus	9.0	6.0	10.2	0.11
Methicillin-resistant *S. aureus*	10.0	6.4	11.2	0.13

Ciprofloxacin was used as positive control. *S. aureus: Staphylococcus aureus*

Molecular modeling

The protein structural file of CrtM (protein data bank code 3ACX) was downloaded from the PDB (www.rcsb.org).[16] Protein for docking was prepared by utilizing Surflex-Dock tools[17,18] and by adding missing hydrogen atoms, adjusting tautomerization and protonation states of amino acids, and relaxing the protein-ligand clashes. Fixing atom types, ring enumeration, and relaxation steps were performed by applying Tripos force field and Gasteiger-Hückel charges. All ligands were sketched and their protonation, tautomerization, and ionization states were checked in MarvinSketch.[19] To account for protein flexibility in the docking step, the intrinsic dynamics of the protein were checked by calculating the anisotropic network model (ANM) using ProDy tools.[20] Generation of 250 normal modes with a cutoff was created for interaction between Cα of 15 Å, and the 10 most diverse structures were kept for docking. The receptor grid was prepared using the cognate ligand coordinates. In the molecular docking step, application of pre- and post-docking ligand minimization was done for more accuracy. Ligand ring flexing was taken into consideration and allowed for top ten poses for each compound.

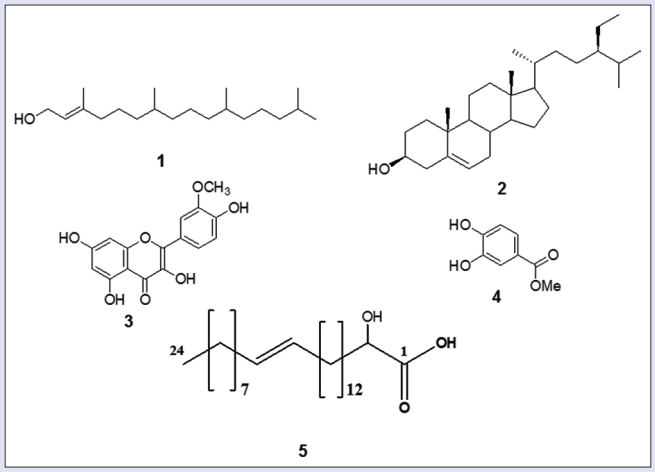

Figure 2: Compounds 1–5

RESULTS AND DISCUSSION

Dried aerial parts of *L. amplexicaule* were exhaustively extracted with ethanol; fractionation on VLC column was performed with *n*-hexane, ethyl acetate, and methanol, respectively. The obtained crude extract was then treated using combined chromatographic separations, and five compounds were isolated [Figure 2]. Their structures were elucidated by different spectroscopic techniques. The isolated metabolites were identified as phytol (1), *β*-sitosterol (2), isorhamnetin (3) 3,4-dihydroxy-methyl benzoate (4), and hydroxynervonic acid (5). The secondary metabolites were evaluated for their antimicrobial activity. Compounds 1, 3, and 4 showed activities against MRSA with IC_{50} values of 10.0, 6.4, and 11.2 μg/mL, respectively, and also exhibited activity against standard *S. aureus* with IC_{50} values of 9.0, 6.0, and 10.2 μg/mL, respectively. Compounds 2 and 5 exhibited no activity against tested organisms.

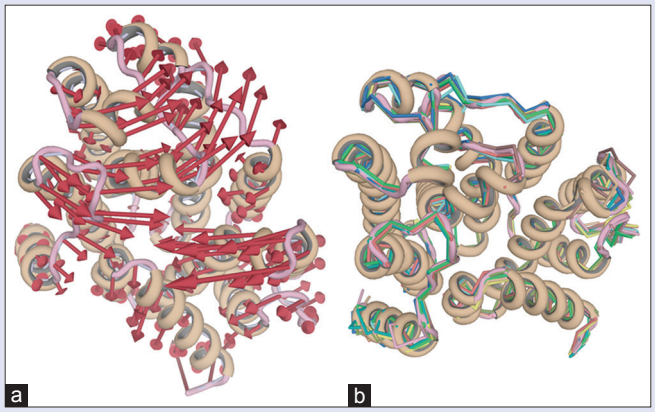

Figure 3: CrtM structure showing the first five normal modes. (a) The arrows represent the degree of Cα fluctuations. (b) Different normal modes are shown as ribbons. α-Helices are colored as wheat and loops as pink

It is expected that the active compounds are interfering with STX virulence factor biosynthesis in *S. aureus*. The intrinsic dynamics of CrtM were described by applying ANM calculations. The normal modes of CrtM suggested wide flexibility at the ligand binding site [Figures 3 and 4]. Several amino acid residues are found to be involved in Van der Waals, electrostatic or hydrogen boning interactions with the cognate ligand including Met15, His18, Phe22, Phe26, Val37, Tyr41, Cys44, Arg45, Asp48, Val133, Ala134, Val137, Leu141, Leu145, Ala157, Leu160, Leu164, Gln165, Ile241, and Phe233. We used Surflex-Dock to prepare the receptor grid using the apo-structure and using the cognate ligand coordinates in two different steps, and we obtained the same pocket [Figure 5], which explains the drugability of the ligand binding pocket. The receptor grid was found to contain many hydrophobic points that are believed to be important for the enzyme function. CrtM binds to presqualene diphosphate, which is a hydrocarbon with a high degree of hydrophobicity. Furthermore, the receptor contained polar points represented by nitrogen atoms that are needed for the interaction with the diphosphate moiety. We checked the binding of our compounds to CrtM, and the results were consistent with the experimental findings. The compounds showed better docking scores with 3 than the others. The polar atoms are found to be aligned well in the polar points of the receptor and the carbon atoms are aligned to the hydrophobic regions. This explains in part the slight better activity for 3. The two other structures are less balanced in terms of hydrophobic to hydrophilic atoms making the alignment to the receptor points less favorable with comparison to 3. These structures and in particular 3 could be used as lead to synthesize new active CrtM inhibitors which are highly needed to manage *S. aureus* nosocomial infection.

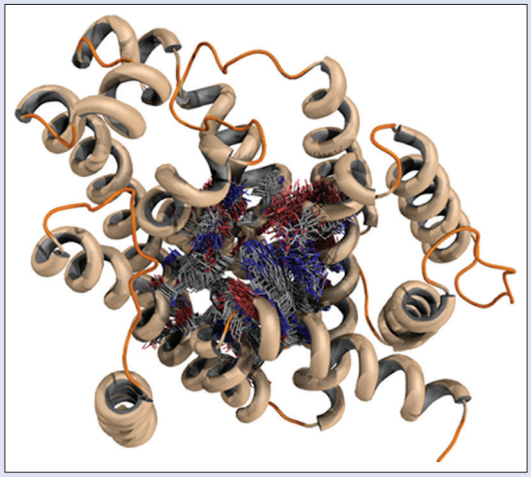

Figure 4: CrtM structure. The high degree of flexibility at the ligand binding pocket is represented by the multiple states of each amino acid residues aligning the pocket

The analysis of the ligand-binding pocket of CrtM revealed its tendency to accommodate hydrophobes with polar groups. The isolated compounds lack the required hydrophobic/hydrophilic balance to exert better activity. These compounds would provide good new scaffolds for anti-MRSA by considering the necessary hydrophobic/hydrophilic balance of the ligand-binding pocket [Figure 6].

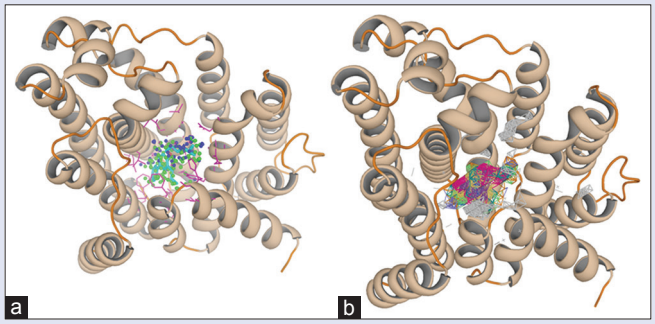

Figure 5: The ligand-binding pocket of CrtM. (a) The cognate ligand is shown as cyan sticks and the surrounding amino acid residues as purple lines. The hydrophobic points of the active site are shown as green spheres (carbon), and the polar points as blue (nitrogen) spheres. (b) The volume of the active site is displayed by a mesh

CONCLUSION

Five compounds were first isolated from *L. amplexicaule*, three of them showed moderate-to-strong anti-MRSA activity. The anti-MRSA activity of these compounds was compatible with the docking studies that prove their mechanism of action.

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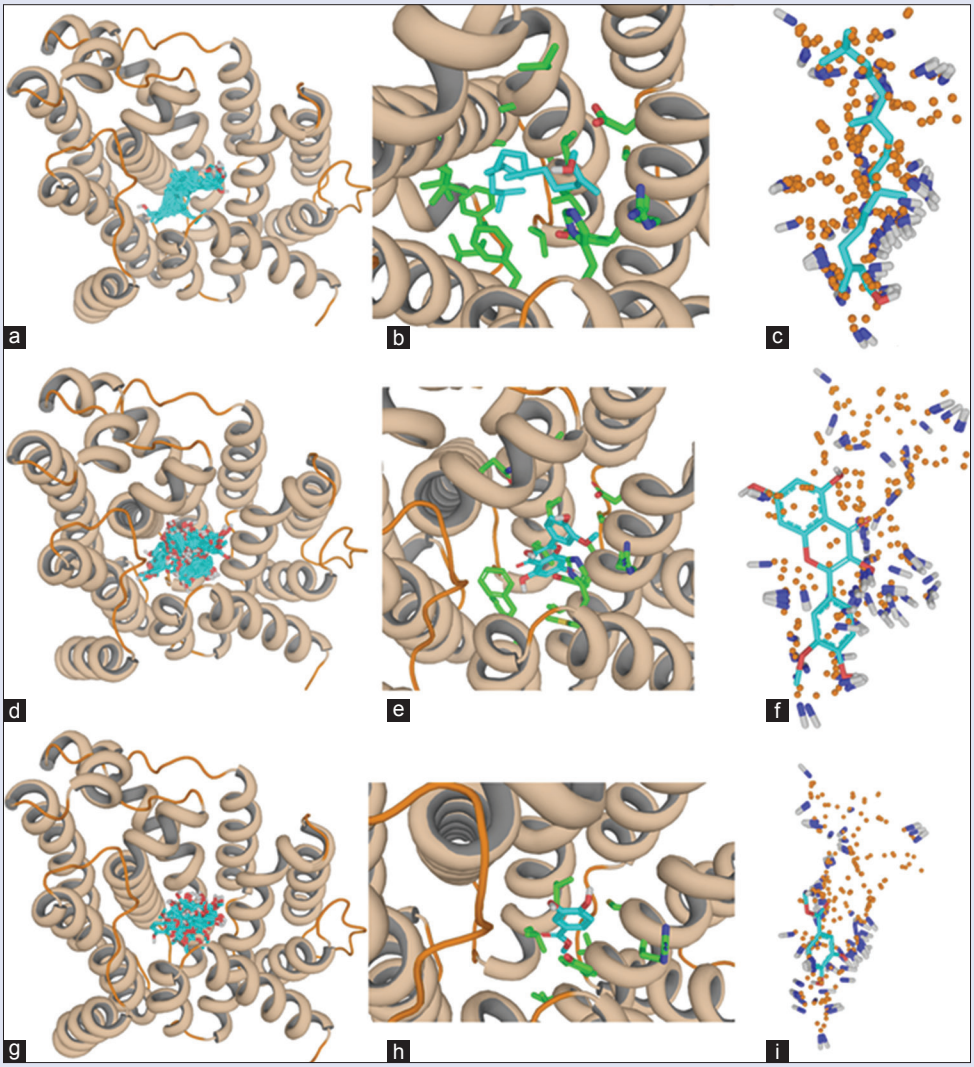

Figure 6: The predicted binding modes of compounds 1, 3, and 4. (a, d, and g) The top ten docking poses of compounds 1, 3, and 4, respectively. (b, e and h) The best binding pose of each compound showing the surrounding amino acid residues. (c, f and i) The best binding mode of each compounds interacting with the hydrophobic (orange) and hydrophilic (blue) points of the active site

Conflicts of interest

There are no conflicts of interest.

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