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RESEARCH ARTICLE

The mechanism of action underlying antibacterial activity of a diterpene quinone derivative against *Staphylococcus aureus* through the *in vitro* and *in silico* assays

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Abstract

Background: The need for new antibacterials to combat resistance is still constrained by the availability of antibacterials that are safe to use. Diterpene quinone-derived compounds are proven to have antibacterial activity. However, their mechanism of action is unknown. **Objective:** This study aims to determine the mechanism of antibacterial action of a diterpene quinone (6O7AR) on *Staphylococcus aureus* through *in vitro* and *in silico* assays. **Method:** MIC tests were performed using microdilution, and MBC determinations were carried out on MHA media. *In vitro* assays were conducted using membrane permeabilizing with Tris, Triton X-100, and ATPase-inhibiting agents with NaN3. Docking was performed on 2XCT proteins in *S. aureus* bacteria using AutoDock Vina. **Result:** The MIC and MBC results of 6O7AR were 300 µM and 2400 µM, respectively. The *in vitro* assay result suggested that the antimicrobial activities of 6O7AR were associated with the inhibition of ATPase function and disrupting membrane function. The docking results showed that the compound possessed good interactions with the 2XCT proteins of *S. aureus*. **Conclusion:** 6O7AR exhibited good antibacterial activity. Based on *in vitro* and *in silico* assays, the mechanism of action of this compound is related to the disruption of bacterial membrane function, and it has the potential to inhibit the ATPase enzyme and the *S. aureus* gyrase.

Introduction

Infectious diseases are still a common health problem in developed and developing countries. Bacteria, both Gram-positive and Gram-negative bacteria, are the most common microorganisms that cause infections (WHO, 2017a). Recent research by the Global Burden of Disease 2019 team, released in 2022, revealed that the second most deaths in the world are caused by bacterial infections. There were 33 commonly identified bacterial pathogens and 11 types of infections across 204 countries. These pathogens were associated with 7.7 million deaths, or about 13.6% of all deaths reported worldwide before the COVID-19 pandemic. Of the 33 bacterial pathogens, five caused half of the reported deaths: *Staphylococcus aureus, Escherichia coli, Klebsiella pneumoniae, Streptococcus pneumoniae,* and *Pseudomonas aeruginosa* (GDB 2019 Antimicrobial Resistance Collaborators, 2022).

Staphylococcus aureus is one of the most common human pathogens that causes various clinical illnesses. It is a primary cause of infections linked to devices, pleuropulmonary, osteopathic, skin and soft tissue,

and bacteremia, as well as infective endocarditis. The widespread use of antibiotics for the treatment of these conditions has led to the emergence and spread of drug resistance to *S. aureus* strains (Adwan *et al*., 2009). Antibiotics are administered properly in order to overcome bacterial infections (WHO, 2017a). The discovery of new antibacterial agents is undeniably still one of the interesting topics to explore, where the need for new antibacterial agents to combat resistance is still constrained by the availability of antibacterials that are safe to use. The irrational use of antibiotics in various fields of medical science is one of the causes of acquired resistance (Soleha, 2015). On the other hand, the use of antibiotics also often causes side effects such as allergic reactions, idiosyncratic reactions, toxic reactions, and biological and metabolic changes in the host (Gunawan, 2012). This situation encourages to look for alternative treatments that are relatively more effective and safer, including the use of drugs isolated from natural ingredients. Plants have active substances that can be used as antibacterial agents. Therefore, it is very important to find new antibacterial agents to overcome resistance (Darmawijaya, 2018).

The use of herbs for medicinal purposes is one of the oldest forms of medicine in the world. World Health Organisation (WHO) predicts that about 80% of the world's population has used medicinal plants (herbal medicine, phytotherapy, phytomedicine or botanical medicine) for their health care (WHO, 2017b). According to the results of the Indonesian basic health research in 2018, it was found that the prevalence of the Indonesian population aged over 15 years who had consumed herbal medicines was 59.12% spread across various regions in Indonesia (Riset Kesehatan Dasar (Riskesdas), 2018). With the biodiversity of Indonesia, there is a wide chemical diversity, especially secondary metabolites. One of the secondary metabolites that can be isolated from natural materials (*Plectranthus amboinicus* (Lour.)) is a diterpene quinone derivative 6-OH,7-AcO-royleanone (6O7AR) (Teruna *et al*., 2020). Diterpene quinone is one of the terpenoid derivatives that shows biological and pharmacological activities, including antibacterial, antioxidant, anti-inflammatory, and cytotoxic activities (Matias *et al*., 2019).

Abdissa *et al.* (2017) reported that diterpene quinone derivatives, especially 6-OH, 7AcO-royleanone (6O7AR), and 6,7-di-OH-royleanone (67DOR), were shown to have a higher inhibition zone than the reference drug (gentamicin) against Gram-positive and Gram-negative bacteria. Quinone diterpene-derived compounds are proven to have antibacterial activity, but the mechanism of action is unknown. One of the methods to predict the mechanism of action and antibacterial activity is the molecular docking method. A Molecular docking study is a structure-based drug design strategy to determine the interaction between ligands or test compounds in target cells or proteins by predicting ligand-protein binding interactions in the hope of strong affinity (Teruna *et al*., 2020). In addition, determining the mechanism of antibacterial action can be done using membrane permeabilisation chemicals (Lee *et al*., 2015). So far, it is not known how the antibacterial mechanism of action on secondary metabolites of diterpene quinone derivatives isolated from natural materials. For this reason, it is necessary to know the antibacterial mechanism of action of diterpene quinone derivatives through *in vitro* and *in silico* assays on *Staphylococcus aureus,* one of the most common infectious bacteria.

Methods

The Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) assays.

The antibacterial activity test was done according to the steps described by Sarker et al. (2007). They used the microdilution method and added resazurin to show how much bacteria were growing. This test was conducted against *S. aureus* ATCC 29213 bacteria. A total of 50 µL of 6O7AR with a concentration of 600 µM and positive control chloramphenicol with a concentration of 200 ppm were pipetted into 96-well microplates containing 50 µL of MHB media. This procedure was repeated three times, and a twofold dilution was done. Next, 10 µL of resazurin was added, followed by the addition of 10 µL of bacteria with OD600nm ~ 0.1 or equivalent to 10^7 CFU/mL (Martins *et al*., 2011). The controls used in this test were four controls: namely positive control, negative control, media control (sterility control), and bacterial growth control. All controls were pipetted into each well of the microplate, then incubated at 37^{o}C for 18 hours. This experiment was done in triplicate. After incubation, the colour changes were seen. Blue indicates no bacterial growth, while pink indicates bacterial growth. This work was done aseptically. MBC determination was carried out on Mueller Hinton Agar (MHA) media. Test suspensions that showed MBC activity were taken using a micropipette as much as 10 µL and dripped on MHA media. Petri dishes containing the media were incubated for 12-24 hours. Media that did not grow with bacteria showed a minimum bactericidal concentration (MBC).

The in vitro antibacterial mechanism of action assay

One of the antibacterial mechanisms of action is by disrupting bacterial cell membrane function. To determine whether the antibacterial activity of 6O7AR is associated with membrane function, the antibacterial compounds were combined with membrane-permeabilising Tris and Triton X-100 or ATPase-inhibiting agents NaN3, which can reduce ATP levels by disrupting the proton electrochemical gradient in bacteria. Membrane permeabilising agents and ATPase inhibitors are added at concentrations that do not inhibit bacterial growth: 0.01% for Tris, Triton X-100, and NaN_3. The MICs of the test compounds used were at concentrations that did not significantly affect the survival of *Staphylococcus aureus* bacteria. Cultures were incubated at 37◦C for 24 h, and growth was evaluated by measuring at a wavelength of 600 nm using a microplate reader (Lee *et al*., 2015).

The in silico molecular docking

The first step was the preparation of the ligand. In this study, 6O7AR was used as a ligand. The two-dimensional structure of the ligand and chloramphenicol, which was used as a positive control, were made using ChemDraw Ultra 12.0 and saved as .dsv file. Furthermore, energy and RMS optimisation were carried out using Chem3D 12.0 and the data results were then saved in .pdb format. After that, AutoDockTools 1.5.6 was run to set the torsion tree, and the ligand was saved in .pdbqt format. Receptor preparation was carried out by downloading the receptor molecular structure from the Protein Data Bank (PDB) website http://www.rscb.org, namely *S. aureus* gyrase receptor molecules (PDB ID 2XCT). The receptor was downloaded in .pdb format. Then, prepare the receptor using Discovery Studio Visualizer 2021 software. After that, AutoDockTools 1.5.6 was run to adjust the hydrogen and protein charges and then saved in .pdbqt format.

Docking validation was conducted to determine the accuracy of the docking parameters used in the software. Validation was done through redocking, where the natural ligands (CPF) from protein 2XCT were docked back to their active site. Docking was performed using the results of the prepared ligand. The best conformation from docking was selected and then exported into the receptor structure stored in .pdbqt format. Docking was performed using AutoDock Vina. Furthermore, the receptor-ligand complex interaction was visualised using Biovia Discovery Studio Visualizer 2021 software.

Results

The Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) assay

The compound 6O7AR was tested against *S. aureus* ATCC 29213 bacteria. MIC test aims to determine the lowest concentration of the compounds that can inhibit the growth of *S. aureus* bacteria. The test was conducted using five concentrations: 600 µM, 300 µM, 150 µM, 75 µM, and 37.5 µM. The compound that is active in inhibiting bacterial growth is continued with the MBC assay. The test was carried out using four concentrations, namely 2400 µM, 1200 µM, 600 µM, and 300 µM. Antibacterial MIC and MBC results of 6O7AR can be seen in Table 1.

Table I: MIC and MBC test results of 6O7AR compound against *S. aureus* ATCC 29213 bacteria

	6O7AR	
	MIC (µM)	MBC (µM)
S. aureus (ATCC 29213)	300	2400

The in vitro antibacterial mechanism of action of 6O7AR

The antibacterial activity of the diterpene quinone derivatives is enhanced by membrane-binding agents and ATPase inhibitors. To determine the effect of membrane permeability on compounds 6O7AR antibacterial activity, the *S. aureus* ATCC 29213 strain was treated with the combination of 6O7AR (75 µM) and membrane-permeabilising agents Tris (0,01%) and Triton X-100 (0,01%). In the used concentration, Tris, Triton X-100 alone did not significantly affect *S. aureus* growth; however, 6O7AR + Tris and 6O7AR + Triton X-100 decreased *S. aureus* growth by 25% and 28%, respectively, compared to 6O7AR alone (Figure 1a). In addition, 6O7AR (75 µM) was combined with the ATPase inhibitor Nan3 (0.01%). In the concentration used, NaN3 alone did not significantly affect *S. aureus* growth. However, 6O7AR + NaN3 decreased *S. aureus* growth by 20%, respectively, compared to 6O7AR alone (Figure 1b).

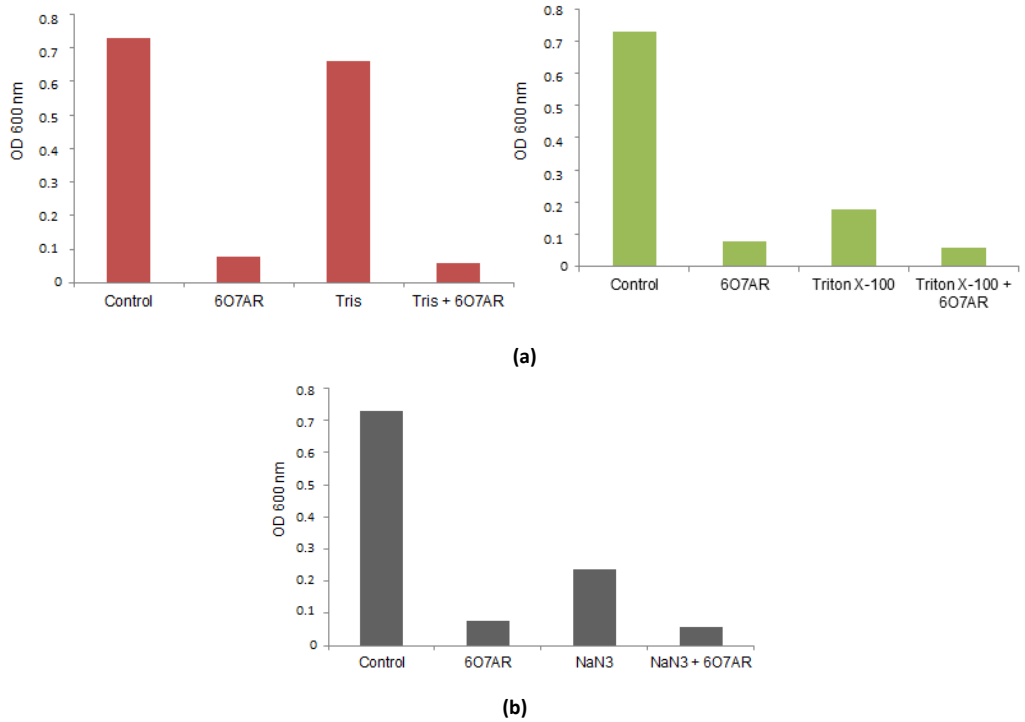

Figure 1: (a) The effect of membrane permeabilising agents on the susceptibility of S. *aureus* (ATCC 29213) to 6-OH, 7AcO-royleanone (6O7AR). Bacterial viability was determined at 600 nm after incubation for 24 h with 6O7AR (75 µM), 0,01% Tris and 0,01%Triton X-100, and their combination 6O7AR + Tris and 6O7AR + Triton X-100. (b) The effect of ATPase inhibitor on 6-OH, 7AcO-royleanone (6O7AR). Bacterial viability was determined at 600 nm after incubation for 24 h with 6O7AR (75 µM), 0,01% NaN3, and a combination of 6O7AR + NaN3. The data is presented as the mean of the three independent experiments. Control, untreated control S. *aureus*.

The in silico molecular docking of 6O7AR

The results of the molecular docking analysis demonstrated that compound 6O7AR and chloramphenicol (CLR) interacted with the *S. aureus* gyrase receptor molecules (2XCT) in an excellent manner. This case was based on their binding affinity, RMSD, inhibition constant, and bonding similarity. The results are presented in Table II and Figure 2.

Table II: Docking Result of 2XCT to CLR and 6O7AR

Complex molecule	Binding affinity (kcal/mol)	RMSD	Inhibition constant (µM)	Parameter						
				Hydrogen bond	Carbon hydrogen bond	Van der Waals	π - Sigma	π - alkyl	π - π stacked	Halogen
2XCT X CLR (positive control)	-7.7	1.04	2.23	**DG (G:9)**, **Asp (B:437)**, **DT (E:8)**, Ser (B:1084)	-	**Arg(B:458)**, DT(G:10), **Gly (B:1082)**, **Gly(B:459)**, DC(H:12)	-			DA (H:13)
2XCT X 6O7AR	-7.3	1.47	4.39	**Asp (B:437)**, **DG (G:9)**	Gly (B:436)	**Arg(B:458)**, **Gly(B:1082**, **Gly(B:459)**, Ser(B:438), Ala(B:439), Glu(B:435), Asp(B:508), Asp(B:510), Gly((B:582), His(B:1081).	DG (G:9)		DT (E:8)	-

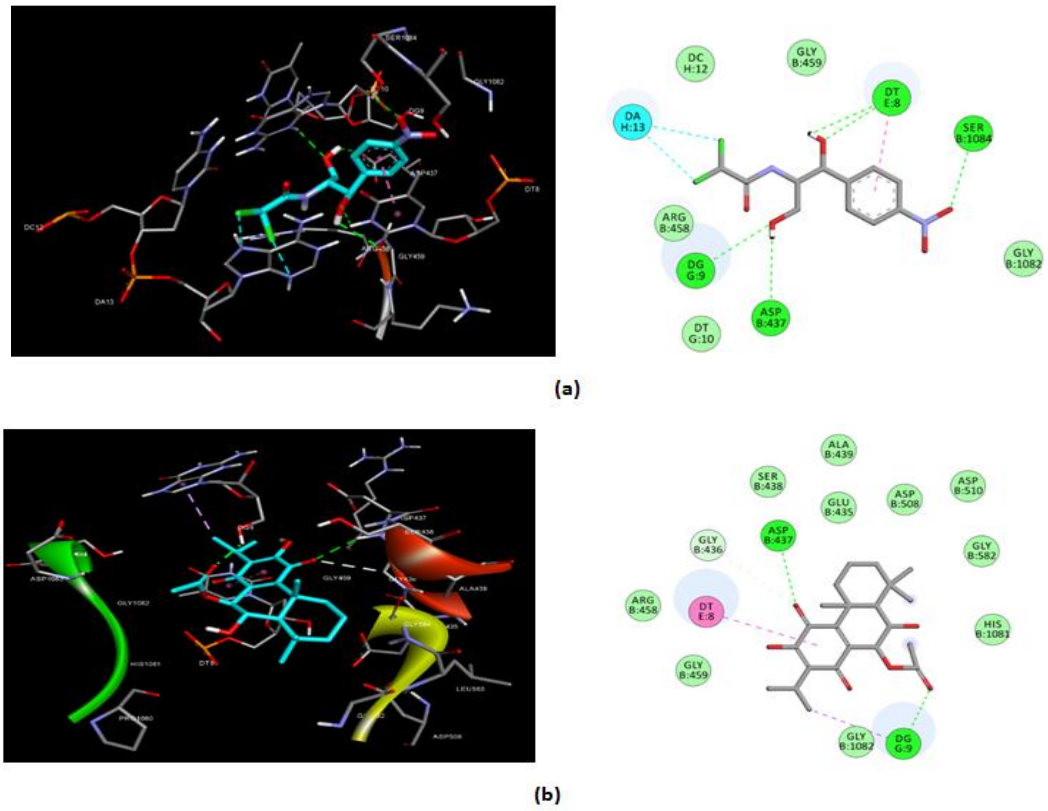

Figure 2: Spatial arrangement at the active site (2XCT), the interaction of CLR (a) and 6O7AR (b) with the protein, visualised using Biovia Discovery Studio Visualizer 2021 software

Discussion

The antimicrobial activity tests on compound 6O7AR were carried out by the microdilution method. The selection of this technique is because it has several advantages, including being able to analyse several different samples at once, using small amounts of samples, requiring a short time, more accurate, and having high sensitivity (Klančnik *et al*., 2010). The addition of resazurin to the test process can predict the presence of antimicrobial activity visually through colour changes. A compound has activity if the results obtained are blue while having no activity or weak activity is marked by a change in colour to pink. This is because blue resazurin is reduced to pink resofurin by the oxireductase enzyme in living cells (Sarker *et al*., 2007). From the MIC test results obtained (Table I), the minimum concentration of 6O7AR compound to inhibit *S. aureus* bacteria (ATCC 29213) is 300 µM. It can be seen that 6O7AR has antibacterial activity. Our findings are consistent with those of a previous study showing that 6O7AR were shown to have a higher inhibition zone than the reference drug (gentamicin) against Gram-positive and Gram-negative bacteria (Abdissa *et al*., 2017).

Plectranthus amboinicus extract has been used in traditional medicine because of its antibacterial, antioxidant, anti-inflammatory, and anti-tumour activities. However, the mechanisms underlying the 6O7AR antibacterial effect have not been investigated. The mechanism of antibacterial action consists of four types: inhibiting cell wall synthesis, disrupting membrane function, inhibiting enzyme action, and preventing the formation of nucleic acids and proteins (Yan *et al*., 2021). In this study, 6O7AR significantly reduced the growth of *S. aureus* at concentrations much lower than MIC when used together with membrane-permeabilising compounds or ATPase inhibitors. It has been demonstrated that Tris and Triton X-100 increase cell membrane permeability and promote cell autolysis (Komatsuzawa *et al*., 2000). The membrane permeabilising activity of Tris and Triton X-100 increased the susceptibility of *S. aureus* to 6O7AR compared to 0.01% Tris or 0.01% Triton X-100 alone.

NaN3 is an inhibitor of ATP synthase in bacterial cells. It is known that NaN3 can interfere with the electrochemical proton gradient in bacterial cells, thereby inhibiting ATP synthesis in mitochondria and ATP-dependent transport processes such as

endocytosis. These results indicate that the anti-*S.aureus* effects of 6O7AR are potentiated by NaN3, suggesting that the antimicrobial activity of 6O7AR is associated with the inhibition of ATPase function. The mechanisms of action assay of compound 6O7AR as antibacterial *S. aureus* was carried out *in silico* with the molecular docking method. Molecular docking is a computational method that predicts the most likely orientation of one molecule when bound to another to form a stable complex (Lengauer & Rarey, 1996). The goal of molecular docking is to achieve the most optimal conformation for proteins and ligands with the least system-free energy. Tests were conducted on proteins in *S. aureus* bacteria, namely *S. aureus* gyrase receptor molecules (PDB ID 2XCT).

The docking results are observed parameters consisting of RMSD, prediction of the ligand conformation and its position and orientation in the coordinate area of ligand-protein interaction (usually referred to as pose), and binding affinity value (Meng *et al*., 2011). RMSD needs to be observed to determine the difference between the predicted value and the observed value of the test compounds, which describes the similarity of the interaction that occurs between the protein and the ligand (≤ 2 Å). The binding affinity value will be exponentially proportional to the inhibition constant, which is also determined in molecular docking. The inhibition constant is a value that describes how much concentration is needed to inhibit an active site of the protein (Ortiz *et al*., 2019). Both van der Waals interaction and hydrogen bonding were also employed as supporting parameters to determine the stability of the ligand-receptor complex.

Based on the docking results, 2XCT protein and CLR (chloramphenicol), the positive control, had a binding free energy of -7.7 kcal/mol and an RMSD of 1.04. CLR interacts via hydrogen bonds with DG (G:9), Asp (B:437), DT (E:8), and Ser (B:1084). Protein 2XCT and compound 6O7AR had a binding free energy of -7.3 kcal/mol and an RMSD of 1.47, as shown in Table II. It appears that the binding free energy of CLR has a higher negative value than compound 6O7AR. As a result, 6O7AR has a longer time to bind to the active site of 2XCT. Compound 6O7AR has hydrophobic interactions with the same amino acids as 2XCT (Asp (B: 437), DG (G: 9)). This interaction is used as a parameter to determine the stability of ligand binding to the receptor. The spatial arrangement of 2XCT with CLR and 6O7AR is presented in Figure 2.

Conclusion

The diterpene quinone-derived compound 6O7AR has good antibacterial activity. Based on the *in vitro* assay, the mechanism of action of this compound is related to the disruption of bacterial membrane function and inhibiting ATPase enzymes. The results of the *in silico* assay using molecular docking methods concluded that 6O7AR has good interactions with proteins 2XCT from *S. aureus* bacteria, so the mechanism of action of quinone diterpene derivatives could be associated with the inhibition of *S. aureus gyrase*.

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