J Herbmed Pharmacol. 2024; 13(2): 240-248.

http://www.herbmedpharmacol.com doi: 10.34172/jhp.2024.48170

Journal of Herbmed Pharmacology

Exploring the anti-acne potential of *Muntingia calabura* L leaves against *Staphylococcus epidermidis: In vitro* and *in silico* perspective

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ARTICLE INFO

Article Type:
Original Article

Article History:
Received: 30 July 2023
Accepted: 12 October 2023

Keywords:
Antimicrobial agent,
Natural products,
Drug discovery,
Teicoplanin-associated locus regulator,
Molecular docking

ABSTRACT

Introduction: *Muntingia calabura* is a medicinal plant possessing antimicrobial properties against various bacteria. The purpose of this study was to examine *in vitro* and *in silico* activity of the ethyl acetate fraction of *M. calabura* leaves against the acne-causing commensal bacterium, *Staphylococcus epidermidis*.

Methods: In this study, *M. calabura* leaves were extracted using ethanol and then further fractionated with ethyl acetate. The phytochemicals in the fraction were identified with thin layer chromatography (TLC). The activity of the fraction was then tested in *S. epidermidis* culture using the agar diffusion method. Additionally, the molecular docking of *M. calabura* phytochemicals constituents was simulated to teicoplanin-associated locus regulator (TcaR) of *S. epidermidis*.

Results: The ethyl acetate fraction of *M. calabura* exhibited robust antibacterial activity against *S. epidermidis* culture, resulting in inhibition zones ranging from 5 to 10 mm. The fraction was found to contain flavonoids, saponins, and tannins as identified constituents. Further, during the molecular docking analysis, stigmasterol and 7-methoxyflavone demonstrated binding to TcaR with a lower and comparable binding energy of -7.40 and -6.19 kcal/mol, respectively, compared to the control drug, Penicillin-G (-6.40 kcal/mol).

Conclusion: *M. calabura* has the potential to serve as a valuable source of active phytochemical compounds for addressing acne. Further studies are needed to isolate and evaluate each compound found in *M. calabura* individually against *S. epidermidis*.

Implication for health policy/practice/research/medical education:
Muntingia calabura leaves fraction exhibited *in vitro* and *in silico* anti-bacterial activities against *Streptococcus epidermidis*. This study confirms that *M. calabura* may be a promising candidate for anti-acne drugs.

Please cite this paper as: Sambi AI, Saputra BW, Setiawati A. Exploring the anti-acne potential of *Muntingia calabura* L leaves against *Staphylococcus epidermidis: In vitro* and *in silico* perspective. J Herbmed Pharmacol. 2024;13(2):240-148. doi: 10.34172/jhp.2024.48170.

Introduction

Acne vulgaris, commonly known as acne, is a chronic skin condition characterized by inflammation of the pilosebaceous layer, accompanied by blockage and accumulation of keratin and fat occurring on the skin's surface, primarily occurring on the face, back, and chest (1,2). Globally, around 85% of young adults aged 12-25, approximately 8% of those aged 25-34, and 3% of adults aged 35-44 suffer from varying levels of acne severity (3). While it often regresses in early adulthood, some individuals continue the experience persistent acne in their adult years (4). The condition typically presents with comedogenics and inflammatory lesions including papules, pustules, nodules, and cysts (4). On the skin's surface, commensal microorganisms form a dense community acting as a complex barrier against external insults (5). The three most common bacteria found on the skin are *Propionibacterium*, *Staphylococci*, and *Corynebacteria* (5,6). Imbalances of natural conditions of these cutaneous microbes have been closely associated with the development of acne (5,7).

Even though most of the previous studies investigating

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Propionibacterium acnes have provided evidence of its role in causing acne, recent studies have also supported the involvement of *Staphylococcus epidermidis* in acne pathogenesis (5,6). *S. epidermidis* is a gram-positive, facultative anaerobic, or aerobic bacterium found on the surface of human skin, which is generally non-pathogenic and harmless under healthy skin conditions (8). However, it can become an invasive bacterium and contribute to acne development. *S. epidermidis* population is elevated nearly to 82% in acne-afflicted skin compared to controls (9,10). Moreover, it plays a role in the excretion of free fatty acids, such as palmitic acid, which has been associated with acne growth and severity (11).

Staphylococcus epidermidis virulence is supported by biofilm formation, which acts as a reservoir of antibiotic-resistance genes that can be transferred to other microorganisms. The virulence genes play an important role in bacterial adhesion, colonization, biofilm formation, and fatty acid metabolism (9). A major biofilm component of *S. epidermidis* is polysaccharide intercellular adhesion (PIA) whose expression is encoded by the icaADBC operon, which is regulated by some transcription regulators (12). Currently, antibiotics, such as erythromycin, doxycycline, and clindamycin are employed to treat acne. However, the use of antibiotics can lead to bacteria resistance (13). The urgent issue of antibiotic-resistant bacteria has prompted a re-evaluation of antibiotic treatment. Additionally, there has been a significant decline in the development of new antibiotics in recent times (14). As an alternative approach to acne therapy, utilizing secondary metabolites from natural products is considered sustainable, safe, and effective in killing and inhibiting the growth of *S. epidermidis* that causes acne.

Antimicrobial compounds derived from plant metabolites have garnered significant attention in recent years. *Muntingia calabura* L. is a traditional medicinal plant commonly used as an anti-inflammatory, antioxidant, antipyretic, antimicrobial activities, and natural antiseptic properties (15-19). Various constituents have been extracted from *M. calabura* leaves, including various flavonoid compounds such as flavone, flavanone, and flavan, which are responsible for its antibacterial activities (15,18). In a previous study, *M. calabura* leaves demonstrated an antibacterial effect against *C. albicans*, *S. aureus,* and *P. aeruginosa*, and it was found to contain flavonoids, alkaloids, sterols, saponins, glycosides, and tannins (14). The compound 2',4'-dihydroxychalcone in the ethyl acetate fraction of *M. calabura* leaves exhibited the most significant antibacterial activity with minimum inhibitory concentration (MIC) value of 50 mg/mL and 100 mg/mL against both methicillin-sensitive *Staphylococcus aureus* (MSSA) and methicillin-resistance *Staphylococcus aureus* (MRSA) (20). This compound exhibited not only antibacterial activity but also anticancer activity (21-23). Moreover, stigmasterol, a phytosterol compound in *M. calabura* leaves, inhibited penicillin binding protein 2a (PBP2a) with the lowest binding energy than other compounds, such as tetradecanoic acid, dodecanoic acid, and ethyl ester compounds (24). However, its potential to combat acne-causing bacteria has not been yet well established.

This study was conducted to investigate the antibacterial activity of the ethyl acetate fraction obtained from *M. calabura* leaves against *S. epidermidis* bacteria. Additionally, molecular docking was employed to predict the binding mode and affinity of representative chemical constituents to the teicoplanin-associated locus regulator (TcaR) of *S. epidermidis*. TcaR serves as a transcription enzyme involved in PIA synthesis, and its constitutive expression in *S. epidermidis* significantly influences the outcome of antibiotic treatments (12). Thus, inhibiting the TcaR protein presents a promising strategy for targeted acne medication, particularly *S. epidermidis*-resistant strain.

Materials and Methods

Plant material

Muntingia calabura leaves were collected in July 2022 from Sleman, Special Region of Yogyakarta, Indonesia. The leaf specimens were then identified and authenticated at the Medicinal Plant Garden Laboratory, Faculty of Pharmacy, Sanata Dharma University. The sample was kept in the Pharmacognosy and Phytochemistry Laboratory Herbarium, Sanata Dharma University No. 688/LKTO/Far-USD/X/2022. Subsequently, the leaves were dried using an oven at 50 °C before being ground.

Extraction and fractionation

The extraction method was conducted according to Simamora et al (17). Two hundred grams of *M. calabura* leaf powder were immersed in 2000 mL of 96% ethanol with a 1:10 (w/v) ratio for 24 hours while continuously being stirred. Afterward, the mixture was filtered using filter paper to obtain the first filtrate and residue. The residue was then submerged again with 2000 mL of 96% ethanol solvent for 24 hours. The second mixture was filtered again using filter paper to obtain the second filtrate and residue. Subsequently, the residue was then immersed in the same volume of 96% ethanol solvent for the third time. The first, second, and third filtrates were combined and concentrated using a rotary evaporator with a temperature of 40 °C. Furthermore, the fractionation was designed based on the study by Sufian and colleagues (20). Then, 10 g of ethanol extract from *M. calabura* leaves were dissolved in 50 mL of distilled water and 50 mL of ethanol (1:1), and fractionation was carried out with 100 mL of n-hexane solvent in a separating funnel and allowed to stand until separation occurred between the n-hexane fraction and the water layer. The n-hexane fraction was then separated, and the fractionation process was continued using 100 mL of ethyl acetate to obtain the

ethyl acetate fraction. The liquid ethyl acetate fraction was further concentrated using a rotary evaporator.

Phytochemical profile confirmation

The ethyl acetate fraction of *M. calabura* underwent phytochemical screening using the thin layer chromatography (TLC) method to identify the presence of flavonoids, saponins, and tannins. For the flavonoid test, the mobile phase was chloroform: methanol (1:1; v/v), and a quercetin solution in methanol served as a comparative control in this test. The saponin test was conducted using TLC and used n-butanol: acetic acid: water (4:1:5; v/v) as the mobile phase, while a saponin solution in methanol was used as a control. The tannin test was employed with TLC with a mobile phase of n-butanol: acetic acid: water (4:1:5; v/v), and tannin in methanol was used as the control. To conduct the tests, 2.5 µL of samples and control solutions were spotted onto the TLC plate using a micropipette and then allowed to develop. Subsequently, the plates were sprayed with Citroboric, Liberman Burchard, and $FeCl_3$ for detection of flavonoids, saponins, and tannins and then examined under UV light at 254 nm. The fraction and control retention factors were determined and compared to identify the presence of flavonoids, saponins, and tannins.

In vitro antibacterial assay

The antibacterial activity of *M. calabura* was assessed using an agar diffusion assay, wherein Mueller-Hinton agar (MHA) was served as the medium. *S. epidermidis* suspensions at 0.5 McFarlan were inoculated using the pour plate double layer method on the MHA base layer. Wells were created using a cork borer, and then 20 µL of *M. calabura* ethyl acetate fractions at 20%, 40%, 60%, 80%, and 100%, along with gentamycin as a positive control, were placed into each well. The plates were then incubated at 37 °C for 24 hours, and subsequently, the diameter of the inhibition zones was measured.

In silico molecular docking

To perform control docking of TcaR PDB ID 3KP2 (25), the protein was downloaded from www.rcsb.org, and the ligand was extracted using BIOVIA Discovery Studio 2021. AutodockTools 1.5.7 was utilized to prepare the ligand, assigning Gasteiger charges, while the protein was protonated and assigned Kollman charges (26). The grid box was set with the center of coordinates at x = 25.864, y = -30.907, and z = -3.622, with a grid size of 40 × 40 × 40, and a spacing of 0.375 Å between grid points. AutoDock 4.2 was used, applying the Lamarckian Genetic Algorithm (LGA) or 100 iterations. The free energy of binding was calculated as a sum of various energy types. Visual analysis of docking was posed by BIOVIA Discovery Studio 2021, ensuring that the RMSD value between initial and post-docking poses did not exceed 2.0 Å (27-29). To retrieve new ligands for docking, PubChem was then prepared and docked using the same parameters as those used in the control dockings.

Results

This study emphasized the antibacterial activity of *M. calabura* leaves, especially against acne-causing bacterial *S. epidermidis* through a diffusion assay. To identify the active compounds qualitatively, this study employed molecular docking simulations to predict their molecular mechanisms. (Figure 1). To maintain the compound's integrity during extraction, fresh leaves were dried, powdered, and then subjected to ethanol maceration to produce viscous and dark brown extract. A lighter-colored fraction was obtained from a liquid partition with ethyl acetate which indicated a specific compound with similar polarity in the fraction (Figure 2a).

Subsequently, we conducted phytochemical profile confirmation to determine the presence of flavonoids, saponins, and tannins in the fraction compared to a control group. These compounds exhibited pharmacological activity in previous studies (14,15,30). The results are presented in Figures 2b, 2c, and 2d, while

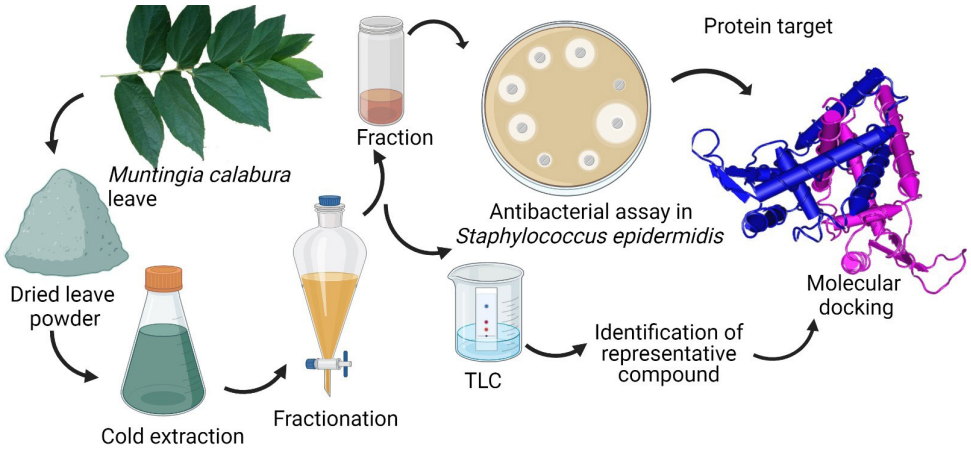


Figure 1. Study scheme of *in vitro* and *in silico* evaluation of *Muntingia calabura* against *Staphylococcus epidermidis*.

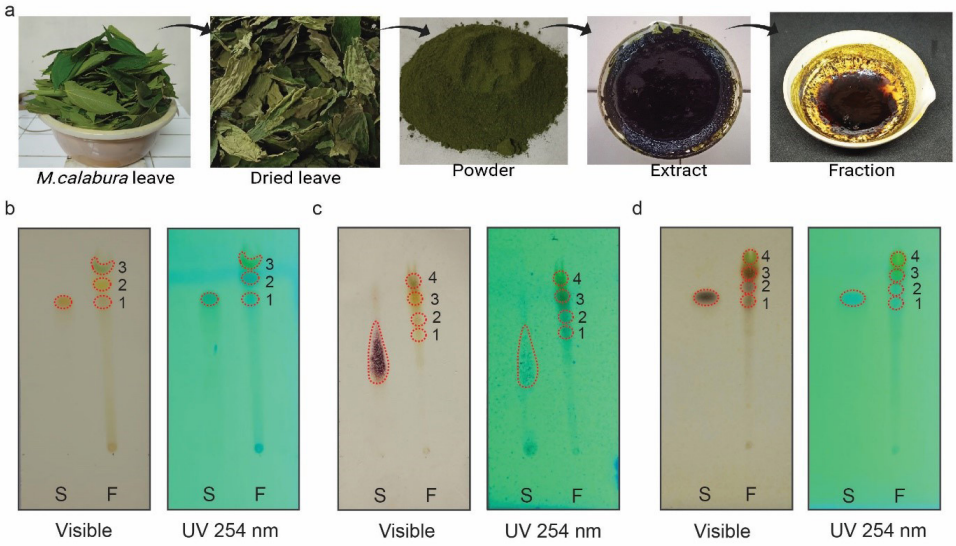


Figure 2. Phytochemical compound isolation and identification. (a) Steps of extraction and fractionation of *Muntingia calabura* leaves from fresh leaves to be an ethyl acetate fraction. Thin Layer Chromatography (TLC) result of Flavonoid (b), Saponin (c), and Tanin (d) under visible and UV 254 nm light after treated spray reagent. The dashed-line area refers to predicted compounds. S: standard compound, F: fraction.

the retention factors of the spots are provided in Table 1. For the flavonoid test, using a mobile phase of chloroform: methanol (1:1, v/v), we observed three spots with R_f values of 0.66, 0.73, and 0.8 in comparison to the control, which had an R_f value of 0.66. The saponin and tannin tests were performed using a mobile phase of n-butanol: acetic acid: water (4:1:5, v/v). The saponin test exhibited four spots with R_f values of 0.46, 0.53, 0.66, and 0.8, whereas the control had an R_f value of 0.53. Similarly, the tannin test revealed four spots with R_f values of 0.66, 0.72, 0.8, and 0.86 compared to the control, which had an R_f value of 0.66.

Furthermore, in this study, preliminary antibacterial activity was performed using the agar diffusion method using *S. epidermidis* culture. We evaluated antibacterial activity by measuring the inhibition zone of each group of fraction concentrations. The obtained inhibition zone values were 10.53 ± 0.15 mm, 13.57 ± 0.50 mm, 15.40 ± 0.36 mm, and 16.63 ± 0.42 mm for 20%, 40%, 60%, 80%, and 100% w/v of ethyl acetate fraction, respectively (Figure 3a and 3b). Although these values were still significantly lower than the value of the inhibition zone of gentamicin (40 mg/mL), which was 29.43 ± 0.12 mm (P <0.001) (Figure 3b), the fraction exhibited a robust ability to suppress the *S. epidermidis*. Therefore, it is intriguing to further investigate *in silico* experiments to predict active compound action on pathogens involving protein.

Furthermore, an *in silico* study was conducted through molecular docking to evaluate the binding affinity of three representative compounds of *M. calabura*, namely 7-methoxy flavone, 4-methyl-hydroxy benzoate, and stigmasterol (Table 2), to TcaR using AutoDock 4.2. These compounds were previously identified in the ethyl acetate fraction of *M. calabura* (29). To validate the docking parameters, the native ligand (Penicillin-G), positive control of antibiotics, was also re-docked for TcaR, resulting in an RMSD of 1.53 Å (<2.0 Å) (Figure 4), indicating the reproducibility of the docking.

Table 1. Retention factor of ethyl acetate fraction of *Muntingia calabura* on thin layer chromatography (TLC)

Types of phytochemical constituents	Retention factor of comparative control	Retention factor of fraction sample
Flavonoid	0.66	Rf_1 0.66 Rf_2 0.73 Rf_3 0.8
Saponin	0.53	Rf_1 0.46 Rf_2 0.53 Rf_3 0.66 Rf_4 0.8
Tannin	0.66	Rf_1 0.66 Rf_2 0.72 Rf_3 0.8 Rf_4 0.86

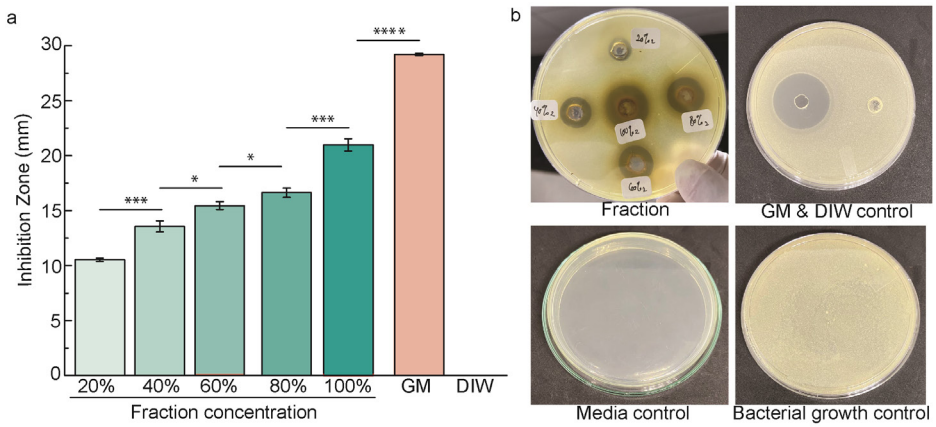
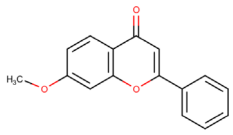
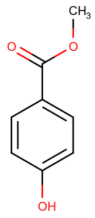
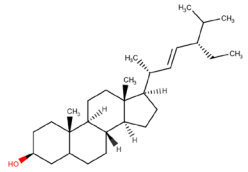
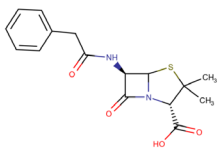


Figure 3. Antibacterial activity of the ethyl acetate fraction of *Muntingia calabura*. (a) Inhibition zone of *M. calabura* fractions against *S. epidermidis* at different concentrations, gentamycin (GM), and distilled water (DIW). Results are presented in means ± standard deviation (SD) (n=3). The statistical significance was assessed a one-way ANOVA followed by the Tukey test. The significance levels were denoted as $^{*}P < 0.05$, $^{***}P < 0.005$, and $^{****}P < 0.001$; (b) Image of *Muntingia calabura* leaves fractions on controls based on well-diffusion assay.

Following the validation process, the molecular docking of three compounds was performed, and the results were presented in Table 2, Figure 5, and Figure 6. Figure 5 illustrates the superimposition of 4 ligands in the active site of TcaR. Table 2 displays the free energy of binding (ΔG_{bind}) and the amino acids that interact with the ligand, while the 3D binding poses of each ligand are shown in Figure 6. A smaller ΔG_{bind} indicates a stronger interaction between the ligand and the protein target, and stigmasterol exhibited the smallest ΔG_{bind} value. It interacted with TcaR through one hydrogen bond (Arg110), van der Waals interactions (Thr23, Leu27, Gln31, Glu39, Ser41, Gln61, Val63, Ala67, Arg71, Tyr106), and hydrophobic interactions, including alkyl (Val43) and pi-alkyl (Ala24, Ala38, His42). Stigmasterol exhibited a better interaction than the native ligand (Penicillin-G), indicating that stigmasterol may serve as a more effective TcaR-DNA interaction inhibitor. Additionally, 7-methoxy flavone emerged as a promising

Table 2. Molecular docking results of three phytochemical constituents from *Muntingia calabura* fraction and control ligand which was co-crystalized with TcaR (PDB ID 3KP2)

Ligand	Chemical Structure	ΔG_{bind} (kcal/mol)	Type of interaction and interacted amino acid residues
7-Methoxy flavone		-6.19	H-bond (Ser41) Pi-Sigma (Ala24) Pi-Alkyl (Ala24) van der Waals (Asn20, Thr23, Leu27, Gln31, Ala38, Glu39, His42, Asn45, Gln61, Arg110)
Methyl 4-hydroxybenzoate		-4.73	H-bond (Asn20, Ser41, Arg110) Akyl (Ala24) Pi-Alkyl (Ala38) van der Waals (Thr23, Leu27, Gln31, Glu39, His42, Asn45, Gln61)
Stigmasterol		-7.40	H-bond (Arg110) Akyl (Val43) Pi-Alkyl (Ala24, Ala38, His42) van der Waals (Thr23, Leu27, Gln31, Glu39, Ser41, Gln61, Val63, Ala67, Arg71, Tyr106)
Control ligand (penicillin G)		-6.40	Ionic (Arg110) H-bond (His42) Akyl (Ala24, Leu27, Ala38) Pi-Anion (Glu39) van der Waals (Asn20, Thr23, Gln31, Ser41, Val43, Asn45, Gln61, Arg71

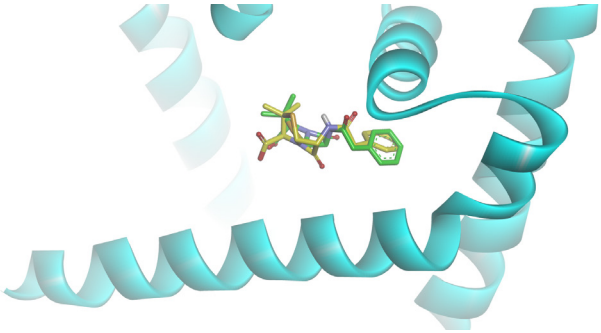


Figure 4. The superimposition of the initial (yellow) and re-docked (green) pose in the active site of TcaR (PDB ID 3KP2).

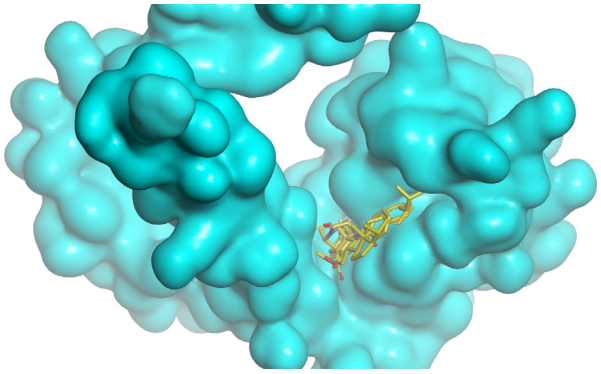


Figure 5. The superimposition of all the docked compounds docked to the active site of TcaR (PDB ID 3KP2). The protein was presented as surface and visualized using BIOVIA Discovery Studio 2021.

candidate for an antibacterial compound against acne-causing bacteria as its binding energy value (-6.19 kcal/mol) was close to that of the control. The 7-methoxy flavone bond to TcaR through hydrogen bond (Ser41, Pi-Sigma (Ala24), Pi-Alkyl (Ala24), and van der Waals interaction (Asn20, Thr23, Leu27, Gln31, Ala38, Glu39, His42, Asn45, Gln61, Arg110) (Table 2).

Discussion

In our study, *M. calabura* leaves were extracted using ethanol and then fractionated with ethyl acetate to isolate the potential active compounds that might be present in the leaves. Based on the empirical application of the leaves, it helps to relieve prostate problems, gastric ulcers, and headaches (31). In some studies *M. calabura* leaves have exhibited antimicrobial effects (14,15,17). Therefore, this study focused on the antimicrobial activity of *M. calabura* leaves against acne-causing bacteria.

Our study revealed a positive result of the ethyl acetate fraction in inhibiting *S. epidermidis* through well-diffusion assay, with inhibition zones ranging from 10 to 20 mm (32). The presence of flavonoids, saponins, and tannins, identified in the fraction, might contribute to this activity; however, the specific compounds in the fraction were not determined in this study. Upadhye et al mentioned that *M. calabura* leaf contains 20,40-dihydroxy chalcone, (2S)-50-hydroxy-7,8.30,40-tetramethoxyflavan, 20,40-dihydroxy dihydrochalcone, 3,4,5-trihydroxybenzoic acid, and 2a,3b-dihydroxy-olean-12-en-28-oic acid (33). A previous study has also been detected triterpenoid steroids, such as stigmasterol and sitosterol, in its semi-polar fraction (15). The presence of these compounds in plants highlights their potential as a source of bioactive phytochemicals, as they may act in synergy (34,35).

Our *in-silico* study docked 7-methoxy flavone, 4-methyl-hydroxy benzoate, and stigmasterol as representative phytochemicals of *M. calabura* leaves, which are flavonoid, phenolic, and saponin. In a previous

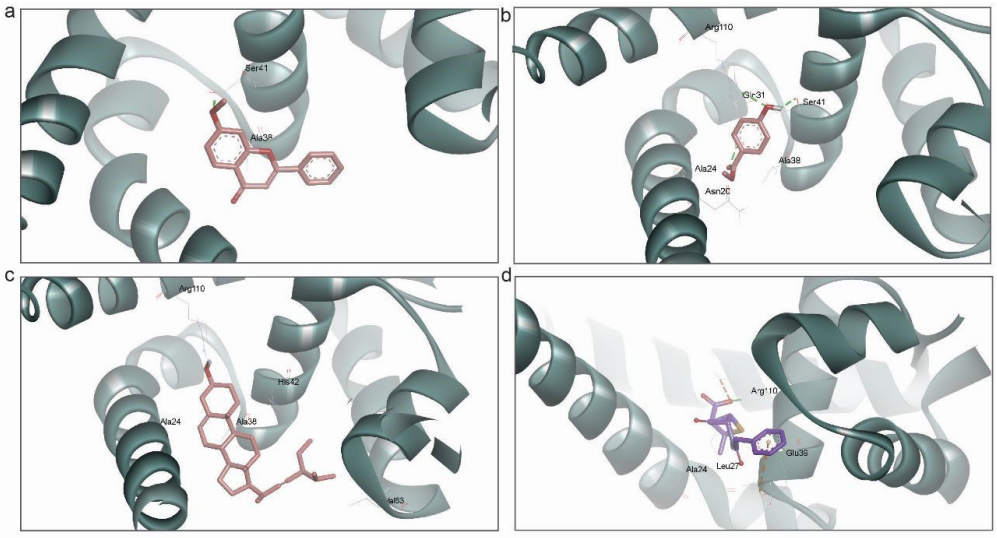


Figure 6. Overlay of best-scored three-dimensional binding poses of *Muntingia calabura* representative compounds to the binding site of Teicoplanin-associated Locus Regulator (TcaR): a. 7-methoxy flavone, b. 4-methyl-hydroxy benzoate, c. stigmasterol, d. Penicillin-G. The hydrogen bonds were shown as green dashed lines.

study 2',4'-dihydroxychalcone in the ethyl acetate fraction of *M. calabura* revealed both antibacterial and anticancer activities (21-23). It inhibited bacterial wall synthesis, specifically in *S. aureus* (23). Since this study focused on TcaR virulence factor in *S. epidermidis,* we excluded 2',4'-dihydroxychalcone from our experiments. Based on our *in silico* study, the presentative compounds of *M. calabura* leave inhibited TcaR, specifically 7-methoxy flavone, and stigmasterol. It suggests that both compounds have higher affinities to TcaR and are more likely to bind tightly, which could lead to a better inhibitory effect. However, to be a potential inhibitor, many other factors also play significant roles. The specific shape of the binding site, the chemical properties of the molecules, and the overall context of the biological system can affect the effectiveness of inhibition or interaction to reduce biofilm formation in *S. epidermidis*.

Multiple antibiotics were found engaged with TcaR at the winged helix-turn-helix motif's binding site. The interaction impeded its binding to the DNA target, resulting in the inhibition of PIA and biofilm expression. The active site of TcaR residues is ARG110, ASN20, HIS42, ASN45, ALA38, VAL63, VAL68, ALA24, VAL43, ILE57, and ARG71. Those amino acids play a promising role in the inhibition process (35). The strongest ligands, stigmasterol and penicillin, bind to the active site of TcaR at the same amino acid residues (ARG110, HIS42, ALA38, ALA24, and VAL43). This result implies that stigmasterol could serve as a likely to have better inhibition activity than commercial antibiotics.

Staphylococcus epidermidis exhibited fundamental results to explore *M. calabura to* overcome resistant strain of *S. epidermidis.* Hence, it might be concluded that the phytochemical compounds of *M. calabura* may modulate or modify the resistance mechanisms in bacteria, suggesting that phytochemicals potentially be proposed in combination with antibiotics to increase the activity and decrease the doses of antibiotics. Taken together, further studies are necessary to evaluate the effect of fraction and antibiotics combination in acne-causing bacteria. Thus, single compound isolation from *M. calabura* and its antibacterial testing is required to evaluate each compound's efficacy toward acne. Additionally, *in vitro* experiments related to the inhibitory mechanism related to TcaR binding, specifically its activity against TcaR-related resistant bacteria, are suggested. It proposes as unfavorable findings emerge the study of compounds derived from plants to be used for medical purposes.

Conclusion

The *in vitro* antibacterial activity of the ethyl acetate fraction of *S. epidermidis* was demonstrated in this study. Phytochemical confirmation using TLC revealed the presence of phenolic, tannin, and saponin compounds in the fraction. Moreover, an *in silico* study of representative phytochemical compounds, namely 7-methoxy flavone, 4-methyl-hydroxy benzoate, and stigmasterol, showed their bindings to TcaR. Among the compounds, stigmasterol exhibited the lowest binding energy. Taken together, *M. calabura* has the potential to be explored as a source of antiacne compound. Further studies are recommended to isolate and test each compound from *M. calabura* against *S. epidermidis*.

Also, the ethyl acetate fraction of *M. calabura* leaves remarkably exhibited strong *in vitro* inhibition activity against *S. epidermidis* by impeding TcaR's activity resulting in reduced biofilm formation. To sum up, *M. calabura* is a potential source of active phytochemical compounds against acne.

Authors' contributions

Conceptualization: Aloisius Imanaldi Sambi, Bakti Wahyu Saputra.
Data curation: Aloisius Imanaldi Sambi, Bakti Wahyu Saputra.
Formal analysis: Aloisius Imanaldi Sambi.
Funding acquisition: Agustina Setiawati.
Investigation: Aloisius Imanaldi Sambi.
Methodology: Aloisius Imanaldi Sambi.
Project administration: Agustina Setiawati.
Resources: Agustina Setiawati.
Software: Bakti Wahyu Saputra.
Supervision: Agustina Setiawati.
Validation: Bakti Wahyu Saputra, Agustina Setiawati.
Visualization: Bakti Wahyu Saputra, Agustina Setiawati.
Writing–original draft: Aloisius Imanaldi Sambi, Bakti Wahyu Saputra, Agustina Setiawati.
Writing–review & editing: Agustina Setiawati.

Conflict of interests

The authors declare no conflict of interest.

Ethical considerations

This study was carried out following the protocols approved by the Ethics Committee of Respati University of Yogyakarta, Indonesia (Approval No. 096.3/FIKES/PL/VI/2023).

Funding/Support

This research did not receive any specific grant project from any funding agencies.

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