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Full Length Article

LC-MS/MS Analysis and Evaluation of Antibacterial Activity of *Curculigo latifolia* Extracts

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Abstract

Acne vulgaris is one of the most common skin problems affecting approximately 9.4% of the global population. It is caused by *Propionibacterium acnes, Staphylococcus aureus* and *Staphylococcus epidermidis.* Extracts from *Curculigo latifolia* Dryand. Ex W.T. Aiton are rich in bioactive substances with antioxidant and antimicrobial properties. This study aimed to identify the active secondary metabolite and to evaluate the antimicrobial activity of *C. latifolia* against *P. acnes, S. aureus* and *S. epidermidis* both *in vitro* and *in silico*. The roots, stems and leaves of the *C. latifolia* plant were extracted by maceration. Each extract was screened for inhibitory activity against the selected bacterial species. The results showed that ethyl acetate extract of leaves significantly inhibited the growth of all the tested bacterial species. LC-MS/MS analysis revealed ten compounds including flavonoids, phenolics and terpenoids. Molecular docking analysis of target proteins of *P. acne, S. aureus* and *S. epidermidis* against the identified compounds showed good interactions observed through free binding energy, inhibition constants and hydrogen bonds parameters. These compounds were 13-apo-beta-carotene; 2-(2,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-chromen-4-one; 2-(2,6-dimethoxyphenyl)-5,6-dimethoxy-4H-chromen-4-one; tangeritin and tetramethylscutellarein. The results of the present study regarding antibacterial activity of ethyl acetate extract of *C. latifolia* leaves will lead to develop this extract as an antibacterial candidate in alternative acne treatment.

Keywords: Antibacterial; Acne Vulgaris; *Curculigo latifolia extract; Propionibacterium acne*; *Staphylococcus aureus*

Introduction

Acne vulgaris, also known as pimples, affects approximately 9.4% of the global population and is the 8th most common skin diseases in the world (Tan and Bhate 2015). Acne is a skin problem that affects many men and women in Indonesia. It was reported in November 2021 that about 46% of skin problems in Indonesian women of age 15–44 years is acne (Qonnayda and Sutini 2021). Acne is a chronic inflamation of the pilosebacea follicle presenting with clinical features like opened or closed comedos, papule, pustule, nodule and cyst. The actual cause of *Acne vulgaris* is still not clear, but there are some etiologies that involved intrinsic factors (genetic, race and hormones) and extrinsic factors (stress, climate, cosmetic, foods and medicines). Besides, there are four factors in the pathogenesis of acne, which include sebum hypersecretion, abnormal proliferation and keratinocyte differentiation in hair follicle, bacterial colonization and inflamatory response. Acne-causing bacteria are *Propionibacterium acnes*, *Staphylococcus aureus* and *Staphylococcus epidermidis* (Wiraputranto *et al.* 2023).

Acne therapy is based on two key principles, which are to prevent secondary infection and to ameliorate the inflamatory process by using antibiotic such as clindamycin, azithromycin, erythromycin, chlaritromycin, roxithromycin, tetracycline, doxycycline and minocycline (Xu and Li 2019). Acne therapy is associated with issue such as antimicrobial resistance and inadequate antimicrobial activity. For this reason, there is the need for more effective alternatives. Herbal and complementary therapy can be useful in this regard. Natural compounds from different plants have been reported to possess potent antibacterial activity (Kanwal *et al.* 2009; Naz *et al.* 2014; Saeed *et al.* 2023a, b).

C. latifolia cultivated in Indonesia, Semenanjung Malaya and Indo-China region has been found to contains compounds such as curculigine, norlignane, terpenoid, flavonoid, tannin, phenol glycoside, with antioxidant and antimicrobial activities (Hong and Ibrahim 2012; Nasir *et al.* 2021; Nur *et al.* 2022).

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Curculigo sp. is used in many traditional medicine practices as antioxidant, antidiabetic, antitumor, antibacterial, anticancer, antiosteoporosis and wound healing agent. Reasearch has shown that several species of the *Curculigo* genus have antimicrobial properties. For example, the ethanolic extract of *C. orhioides* is known to be effective as antibacterial agent against Methicillin-Resistant *S. aureus, Pseudomonas aeruginosa, Klebsiella pneumonia* and *Escherichia coli* (Marasini *et al*. 2015; Venkatachalam *et al*. 2017). Similarly, the methanolic extract of *C. recurvata* has been shown to inhibit *Bacillus cereus* (Kabir *et al*. 2016), while *C. latifolia* has antibacterial activity against *Salmonella choleraesuis, Bacillus cereus, Bacillus subtilis, Enterobacter aerogenes, P. aeruginosa, S. aureus, E. coli, Erwinia* sp. and *Klebsiella* sp. and has antifungal activity against *Candida albicans* and *Cryptococcus neoformans* (Hong and Ibrahim 2012; Farzinebrahimi *et al*. 2016; Nasir *et al*. 2021; Wang *et al*. 2021).

The role of *C. latifolia* plant extract as an antibacterial agent in overcoming acne problems has not been widely studied. The initial hypothesis in this study is that the exploration of *C. latifolia* plant extract (roots, stems and leaves) can provide antibacterial activity. This study aimed to evaluate the activity of *C. latifolia* plant extract in inhibiting the growth of *P. acnes, S. aureus* and *S. epidermidis in vitro* and to identify a secondary metabolite profile of the most active extract with LC-MS/MS and continued to investigate the molecular interaction profile of the compound against the target protein through molecular docking.

Materials and Methods

Materials

Culture media/chemicals: Brain heart infusion broth (Oxoid) media, blood agar (Oxoid), nutrient ngar (Himedia), sodium chloride 0.9% (Otsu - NS), *n*-hexane (technical); ethyl acetate (technical), methanol (technical), Mc Farland 3 (Remel), lugol's iodine (Merck), safranin (Merck), gentian violet (Merck), ethanol 96% (Brataco), aquadest (Brataco), clindamycin (BPOM), sheep blood, hydrochloric acid (technical) and sodium hydroxide (technical).

Organisms: Typed cultures of *Propionibacterium acnes* (ATCC 11827), *Staphylococcus aureus* (ATCC 25923) and *Staphylococcus epidermidis* (ATCC 12228) were obtained from the Microbiology Laboratory, Faculty of Pharmacy, University of Indonesia.

Proteins: Ligand Binding Domain (LBD) 3D protein structure of *Propionibacterium acnes* (PDB ID: 3V3V), *Staphylococcus aureus* (PDBID: 4CJN) and *Staphylococcus epidermidis* (PDB ID: 3KP2) were obtained from Protein Data Bank.

Plant materials: The leaves, stems and roots of the *C. latifolia* were collected from the Spice, Medicinal and Aromatic Plant Instrument Standard Testing Center (BPSI TROA), Indonesia. The plant material was identified and authenticated at Riset and Inovation Agency, Republic of Indonesia (BRIN) with specimen No. B-132/V/D1.05.07/1/2022.

Extraction: Dried powdered leaves, stems and roots of *C. latifolia* were extracted by successive maceration in various solvent of different polarity including *n*-hexane, ethyl acetate and 70% ethanol. The plant materials (250 g each) were macerated in 2.5 L of *n*-hexane, ethyl acetate and ethanol (70%) successively at room temperature for 24 h. The extract was filtered and the marc was remacerated with the same solvent until a clear filtrate was obtained. The combined extract for each solvent was concentrated using a rotary evaporator at reduced pressure to obtain n-hexane, ethyl acetate and ethanol extracts (Nur *et al*. 2023a, b).

Evaluation of antibacterial activity

Media preparation: The brain heart infusion broth (BHIB) powder (37 g) was gradually dissolved in distilled water and stirred until complete dissolution. The solution was made up to 1000 mL with distilled water. The medium was heated to ensure complete dissolution of all the ingredients. The pH of the medium was adjusted to 7.4 ± 0.2 with HCl or NaOH solution as the case may be. The medium was then sterilized in an autoclave at 121°C for 15 min. The medium was cooled to room temperature and stored for 24 h. Similarly, 40 g of blood agar powder was added distilled, stirred until complete dissolution and made up to 1000 mL with distilled water. The medium was heated for complete dissolution and the pH was adjusted to 7.3 ± 0.2 with HCl or NaOH as the case may be. The medium was sterilized in an autoclave at a pressure of 1 atm and a temperature of 121°C for 15 min. The medium was allowed to cool gradually until it reached a temperature of around 40°C.

Sheep blood agar medium (70 mL) was poured aseptically into petri dishes each containing 15 mL of the medium. The blood agar medium in the petri dishes was allowed to solidify. The medium was stored at room temperature for 24 h to ensure the media's compactness and prevent contamination.

Nutrient agar (28 g) was gradually poured into distilled water, stirred until complete dissolution and made up to 1000 mL with distilled water. The medium was then placed in an Erlenmeyer flask and heated on a hot plate until the medium was thoroughly mixed. The pH of the medium was adjusted to 7.4 ± 0.2 with HCl if too alkaline or NaOH if too acidic. The medium was sterilized in an autoclave at a pressure of 1 atm and a temperature of 121°C for 15 min. After sterilization, the medium was allowed to cool to a temperature of about 40–45°C and then poured into petri dishes with each dish containing 15 mL of the medium. The medium was allowed to solidify and stored at room temperature for 24 h to ensure the medium was perfectly compacted and prevent bacterial contamination.

Bacterial screening/gram staining: Bacterial screening was done by Gram staining to determine the Gram-positive and Gram-negative bacteria. A drop of NaCl was placed on

object glass, the bacterial inoculum was added, allowed to dry, then fixed by passing the object glass through an open flame three times. The bacterial inoculum was stained with gentian violet for about 1 min, then washed with running watter. A drop of lugol's iodine was placed on the object glass with the fixed bacteria and allowed to stand for 60 s and then washed again with running water. The bacterial inoculum was decolorized with ethanol (96%) for about 5-30 s. The glass was washed again with running water and safranin solution was added and allowed to stand for 45–60 s, then washed with running water and dried. The bacteria were then examined under a microscope at 10 × 100 magnification. The Gram-positive bacteria appeared violet and the Gram-negative bacteria appeared pinky red.

Bacterial rejuvenation preparation: The test bacteria were rejuvenated in appropriate nutrient medium for *S. epidermidis* and *S. aureus*, while blood agar medium was used for *P. acnes,* using small innoculum (10 μL) of pure bacteria that has been incubated on agar slant. Thereafter, the bacteria were incubated at 37°C for 24 h. The rejuvenated bacteria were used for the antibacterial activity testing.

Preparation of bacterial suspension: The rejuvenated bacteria were inoculated using an inoculation needle into 5 mL of 0.9% NaCl solution and stirred to make a suspension. The clarity of the suspension was measured in McFarland standard 3 and the concentration was 9×10^8 CFU mL^{-1}. Inoculum of the test bacteria suspension was stirred in 5 mL of 0.9% NaCl and vortexed. The clarity of the bacterial suspension was again measured according to McFarland standard 3. This process was repeated until a concentration of 10^6 CFU mL^{-1} of the bacterial suspension was attained.

Determination of antibacterial activity: The antibacterial activity of the ethyl acetate extracts of the leaves, stems and roots of *C. latifolia* was determined using the disc diffusion method (Kirby-Bauer test). A 20 μL volume of the rejuvenated bacterial suspension was placed in a petri dish containing the appropriate growth media. The bacterial suspension was evenly spread using a sterile cotton swab. Paper discs with a diameter of 6 mm impregnated with the extracts and the positive control (clindamycin) were carefully placed on the surface of the media. The media were incubated at 37°C for 24 h for *S. aureus* and *S. epidermidis* and for 72 h for *P. acnes*. The diameter of the inhibition zone was measured in millimeters using a vernier caliper (Anonimous 1953).

Identification of secondary metabolites using LC-MS/MS

A solution of each extract was prepared by dissolving 2 mg of the extract in 10 mL methanol. Protein precipitation was performed by filtering the extract solution through a cellulose acetate filter (0.45 μm) and air bubbles were removed using a degaser. The sample (1 μL) was injected into a UPLC (Ultra Performance Liquid Chromatography) equipped with a Shimadzu Shim Pack FC-ODS column (2 mm × 150 mm × 3 μm). The UPLC system was connected to a Quadrupole Time-of-Flight (QTOF) mass spectrometer. The mobile phase was methanol-water (9:1) at a flow rate was 0.5 mL/min. The ionization was electrospray ionization (ESI) in the positive mode. The capillary temperature was 350°C and the voltage source of 5 V. Full scan mode in the range 100–5000 m/z was used, while keeping the source temperature at 100°C (Nur *et al.* 2023a).

Moleculer docking evaluation

Ligand structure preparation: The 2D structures of the identified compounds were drawn using ChemDraw Ultra 8.0 programme in ChemOffice v. 8.0 package. The 3D structures of the ligand were built with Chem3D v. 8.0 and saved in mol file format. The 3D structures were optimized with the HyperChem 8.0.10 programme. The geometric optimization process was done by adding H atoms in the Austin model 1 (AM1) force field in the HyperChem programme with an RMS gradient score of 0.001. After optimization, the ligand structure was saved in a (.hin) file format and converted to a (.pdb) file format using ArgusLab v. 4.0.1. in the AutoDock Tools v1.5.7. All hydrogen bonds were added to the Ligand, the Gasteinger charges were calculated and then the non-polar hydrogens were merged. The file was saved in (.pdbq) format. The ligand torsion number was calculated and the selected compound wer saved in the (.pdbqt) file format (Nursamsiar *et al.* 2020; Nur *et al.* 2023c).

Protein preparation: The 3D structures of the target proteins of *Propionibacterium acnes* (PDB ID: 3V3V), *Staphylococcus aureus* (PDB ID: 4CJN) and *Staphylococcus epidermidis* (PDB ID: 3KP2) were presented in BIOVIA Discovery Studio Visualizer v. 24.1.0.23298 programme. For *Propionibacterium acnes*, protein target used was Chain A, while for *Staphylococcus aureus* and *Staphylococcus epidermidis*, Chain B was used. Water molecules and native ligands were removed and the protein was saved in a (.pdb) file format and treated as a receptor. Afterwards, a polarized hydrogen atom was added to the protein structure and the partial charge of each atom was counted using the Kollman algorithm provided in AutoDock Tools v. 1.5.7. Finally, the Chain structure was saved in a (.pdbqt.) file format (Tabassum *et al*. 2023; Sambi *et al*. 2024).

Docking method validation: Validation of the docking method was done to ensure that the chosen docking parameters are used for ligand docking with the target protein. The validation process was done by redocking the native ligand in the active site of the receptor or protein (Nur *et al*. 2024).

Moleculer docking simulation: The molecular docking simulation was done using the AutoDock Tools v. 1.5.7. The Grid dimention and the grid box coordinates (x, y, z) were arranged according to each target protein. This Grid was built to cover the amino acid residues in ligand and target protein interaction. The target protein and grid dimension were saved

in a (.gpf) file format, while the electrostatic potential map result was saved in a (.glg) file format. The docking tools were chosen with the Lamarckian algorithm and saved in a (.dpf) file format. The result of the docking simulation was saved in a (.dlg) file format (Nur *et al.* 2024).

Analysis of the docking simulation: The molecular docking simulation was analyzed using the following parameters; ligand structure orientation, hydrogen bonding, binding free energy and inhibition constant from each ligand binding (Nursamsiar *et al.* 2020; Nur *et al.* 2024). Docking result was visualized using Discover Studio Visualizer v. 24.1.0.23298 to show the interaction of test ligand with amino acid residue of the target protein.

Statistical analysis

Antimicrobial activity testing of each test sample was carried out in triplicate. Data were analyzed and expressed as mean ± SD. Significant differences between samples were analyzed through one-way ANOVA ($P < 0.05$, LSD) using SPSS version 21.

Results

Antibacterial activity of *Curculigo latifolia* extract

The results of the antibacterial activity of the extracts of the leaves, stem and roots of *C. latifolia* are presented in Fig. 1–3 and Table 1. The result showed that the positive control (clindamycin) had the highest inhibition zone diameter of 9.5 mm against *P. acnes*, 8.9 mm against *S. aureus* and 7.5 mm against *S. epidermidis*. This indicated the effectiveness of clindamycin in inhibiting bacterial growth. On the other hand, the negative control showed no antibacterial activity with an inhibition zone diameter of 6.6 cm against *P. acnes, S. aureus* and *S. epidermidis.*

Compounds identified from the LC-MS/MS analysis of ethyl acetate extract of *C. latifolia* leaves

The specific identity of chemical compounds and details about the molecular weight and structure of secondary metabolite compounds identified in the ethyl acetate extract of *C. latifolia* leaves have been carried out using LC-MS/MS. Fig. 4 is a chromatogram of the compounds that have been successfully identified. Information regarding the identified compounds can be seen in Table 2.

Optimized 3D structures of ligands and target proteins

The ligands were categorized into two; native ligands and test ligands. The native ligands were downloaded along with the target protein from RCSB, as shown in Table 3, while the test ligands were the compounds identified from the LC-MS/MS analysis of ethyl acetate extract of the leaf of *C. latifolia* (Table 2).

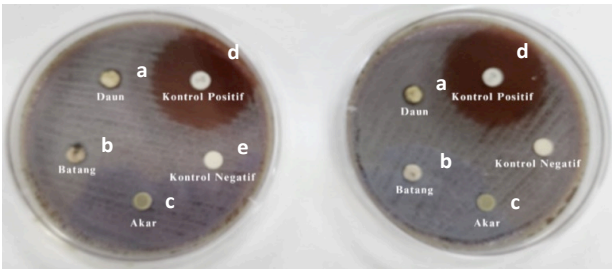


Fig. 1: Antibacterial activity test result of *C. latifolia* extract towards *P. acne.* Leaf extract (**a**), stem extract (**b**), root extract (**c**), postive control (**d**) and negative control (**e**)

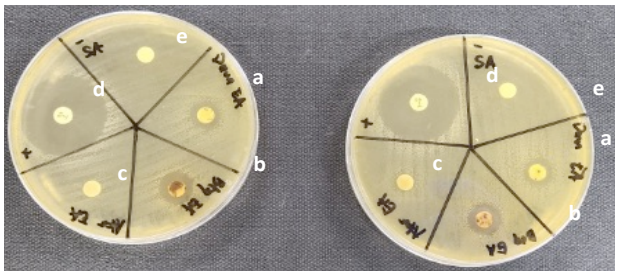


Fig. 2: Antibacterial activity test result of *C. latifolia* extract towards *S. aureus.* Leaf extract (**a**), stem extract (**b**), root extract (**c**), postive control (**d**) and negative control (**e**)

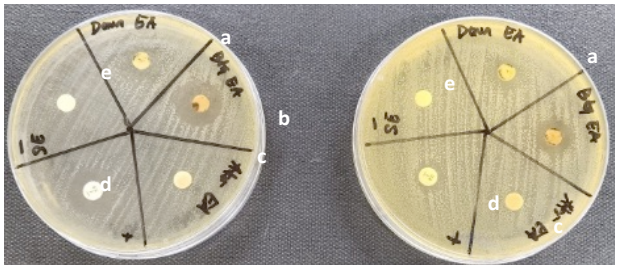


Fig. 3: Antibacterial activity test result of *Curculigo latifolia* extract towards *Staphylococcus epidermidis.* Leaf extract (**a**), stem extract (**b**), root extract (**c**), postive control (**d**) and negative control (**e**)

The target proteins were PDB ID: 3V3V, PDB ID: 4CJN and PDB ID: 3KP2 for *P. acnes*, *S. aureus* and *S. epidermidis*, respectively. The native ligand and water molecules were removed from the protein structure because both can affect the bonding position and affinity score between test ligand and target protein. Polarized hydrogen was added to ensure the right molecular structure for calculating hydrogen bond interaction during the docking simulation, whereas the added Kollman charges were used for calculating the charge distribution in the atoms and molecules for accurate prediction of the interaction during the docking process (Forli *et al.* 2016).

Docking method validation

Docking validation was done by re-docking of each native

Table 1: Antibacterial activity (inhibition zone diameter) of ethyl acetate extract of the leaf, stem and root of *Curculigo latifolia* against *Propionibacterium acne*, *Staphylococcus aureus* and *Staphylococcus epidermidis*

Ethyl acetate extract of *Curculigo latifolia*	Inhibition Zone Diameters (cm)		
	Propionibacterium Acne	*Staphylococcus Aureus*	*Staphylococcus Epidermidis*
Leaf	6.72 ± 0.007*	7.05 ± 0.042*	7.04 ± 0.219*
Stem	6.65 ± 0.014*	7.15 ± 0.042*	7.34 ± 0.262*
Root	6.61 ± 0.014*^{c}	6.62 ± 0.021*^{c}	6.62 ± 0.021*^{c}
Positive control	9.48 ± 0.021*	8.96 ± 0.007*	7.30 ± 0.332*
Negative control	6.6 ± 0.007*^{c}	6.60 ± 0.000*^{c}	6.61 ± 0.021*

Note: * in each row indicates a significant difference between samples ($P < 0.05$), while alphabetical differences indicate no significant difference between the samples

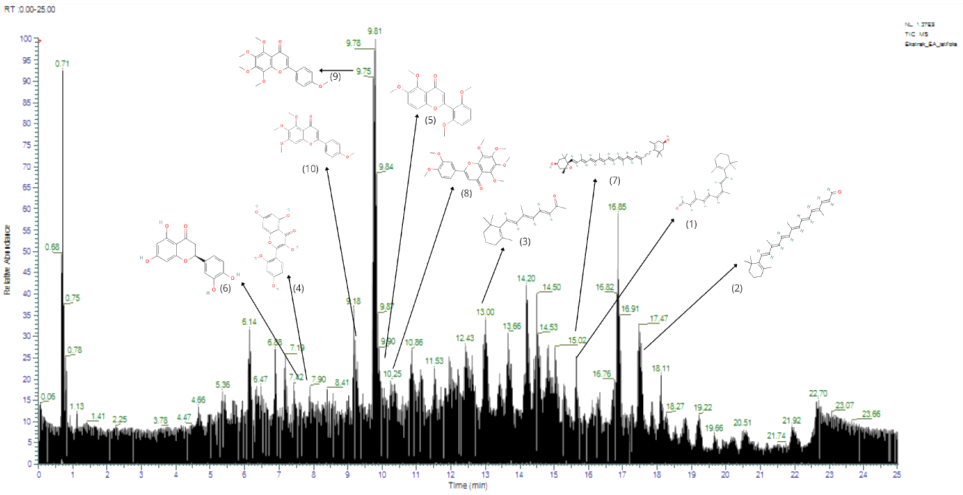


Fig. 4: Chromatogram result of substance in ethyl acetat extract of leaf *Curculigo latifolia*

ligand into the active sites of *P. acnes* (PDB ID: 3V3V), *S. aureus* (PDB ID: 4CJN) and *S. epidermidis* (PDB ID: 3KP2) using gridbox and coordinate as stated in Table 4. The coordinate includes amino acid residues used in the ligand binding. The result of the re-docking was presented in the form of overlay of native ligand position and copy ligand and validated by the Root Mean Standard Deviation (RMSD) score, where a RMSD score less than 2 Å indicate a close similarity between the native ligand and the co-crystallized ligand. The RMSD score, gridbox size and coordinate for each native ligand with the target protein are presented in Table 4.

Moleculer docking analysis

The results of the docking interactions between the test ligands and target proteins: *P. acnes* (PDB ID: 3V3V), *S. aureus* (PDB ID: 4CJN) and *S. epidermidis* (PDB ID: 3KP2) were presented in terms of their binding free energy, inhibition constant and hydrogen bond interaction between the target proteins and ligands (Table 5).

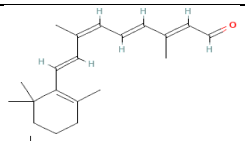
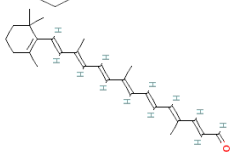
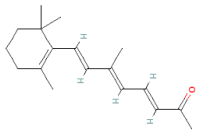
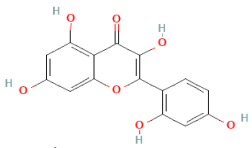
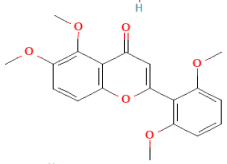
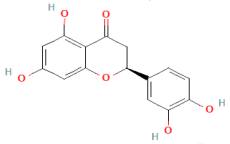
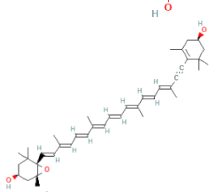
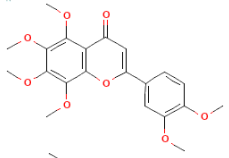
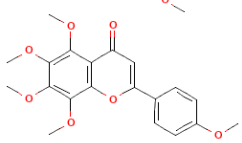
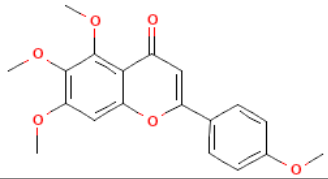
Docking result is visualized using Discover Studio Visualizer v24.1.0.23298 to ligand interaction analysis with amino acid residue of target protein. From tested 10 substances and considering free energy of binding, CI and interaction hydrogen binding, there are 7 substances for *Propionibacterium acne* (PDB ID: 3V3V), 6 substances for *Staphylococcus aureus* (PDB ID: 4CJN) and 5 substances for *Staphylococcus epidermidis* (PDB ID: 3KP2) that more stable than native ligand.

As shown in Table 5, amino acid residues in which there were similar interaction between the native ligand and the test ligand were ASN114 (Asparagine), GLU73 (Glutamic Acid), LEU110 (Leucine) and MET111 (Methionine) for *Propionibacterium acnes* (PDB ID: 3V3V) (Fig. 5). For *S. aureus* (PDB ID: 4CJN) in Fig. 6, the amino acid residues were GLU294 (Glutamic Acid) and LYS316 (lysine), while for *Staphylococcus epidermidis* (PDB ID: 3KP2) the amino acid residue was LYS74 (lysine) (Fig. 7).

Discussion

This study provided information on the bioactivity of *C. latifolia* extract as an antibacterial and the secondary metabolite profile of *C. latifolia* extract, which plays a role in its activity as an antibacterial. The results showed that ethyl acetate extract of *C. latifolia* leaves possess the ability to inhibit the growth of *S. aureus* and *S. epidermidis* growth significantly compared to extracts of other plant parts, but less effective against *P. acnes*. The stem and leaf extracts showed a wider inhibition zone than the root extract, which indicated that some parts of the plant are more effective as antimicrobials than others. The inhibition zone diameter of the extract is far less compared to that of the positive control.

Table 2: LC-MS/MS analysis of ethyl acetat extract of leaf *Curculigo latifolia*

No.	Compounds	Formula	Group compound	Rt (min)	Molecule structure
1	(9 cis)-Retinal	$C_{20}H_{28}O$	Terpenes	15.596	
2	10'-apo-beta-Carotenal	$C_{27}H_{36}O$	Terpenes	17.532	
3	13-apo-beta-Carotenone	$C_{18}H_{26}O$	Terpenes	13.002	
4	2-(2,4-Dihydroxyphenyl)-3,5,7-trihydroxy-4H-chromen-4-one	$C_{15}H_{10}O_7$	Flavonoids	7.593	
5	2-(2,6-Dimethoxyphenyl)-5,6-dimethoxy-4H-chromen-4-one	$C_{19}H_{18}O_6$	Flavonoids	9.924	
6	2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-3,4-dihydro-2H-1-benzopyran-4-one	$C_{15}H_{12}O_6$	Flavanones	7.593	
7	MFCD06656300	$C_{40}H_{54}O_3$	Terpenoid	15.028	
8	Nobiletin	$C_{21}H_{22}O_8$	Flavone	10.256	
9	Tangeritin	$C_{20}H_{20}O_7$	Flavones	9.751	
10	Tetramethylscutellarein	$C_{19}H_{18}O_6$	Flavonoids	9.198	

However, in the inhibitory activity against *S. epidermidis*, the results obtained were impressive, where the ethyl acetate extract of the leaves and stems showed almost the same inhibitory activity as the positive control. This indicates that the extract of the leaves and stems of *C. latifolia* possess strong antibacterial activity against *S. epidermidis* and moderate activity against *S. aureus* and *P. acnes.*

Table 3: 3D Structure of Native ligand and Target protein

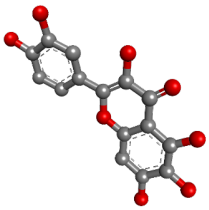
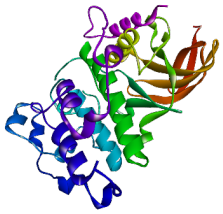
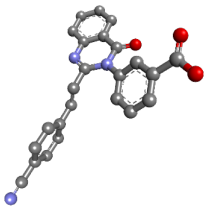
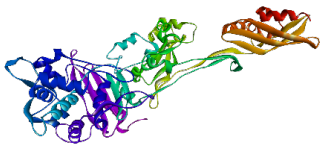
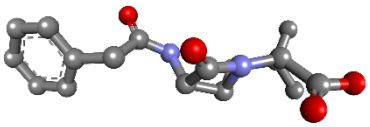
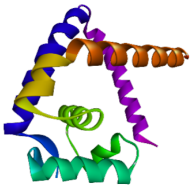
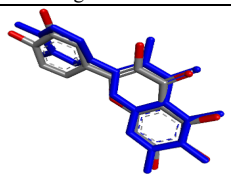
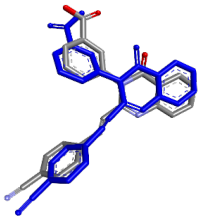
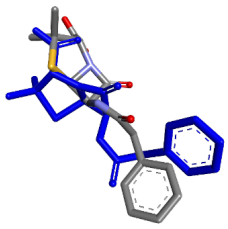
	Native Ligand	Chain Protein
Propionibacterium acne (PDB ID: 3V3V)	 2-(3,4-dihydroxyphenyl)-3,5,6,7-tetrahydroxy-chromen-4-one	 Chain A
Staphylococcus aureus (PDB ID: 4CJN)	 3-[2-[(E)-2-(4-cyanophenyl)ethenyl]-4-oxidanylidene-quinazolin-3-yl]benzoic acid	 Chain B
Staphylococcus epidermidis (PDB ID: 3KP2)	 (2S,5R,6R)-3,3-dimethyl-7-oxo-6-(2-phenylethanoylamino)-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid	 Chain B

Table 4: G*ridbox arrangement* and RMSD score result of hasil docking method validation

Protein	Gridbox	Coordinate (x,y,z)	RMSD (Å)	Native ligand resemble and copy ligand
Propionibacterium acne (PDB ID: 3V3V)	40 × 40 × 40	30.614 44.084 4.212	0.787	
Staphylococcus aureus (PDB ID: 4CJN)	20 × 44 × 34	8.993 -1.203 -69.561	0.953	
Staphylococcus epidermidis (PDB ID: 3KP2)	20 × 14 × 32	28.788 -47.737 -1.319	2.133	

Description: *Overlay* copy ligand in blue coloured

From the antibacterial activity screening of the three extracts, it was found that the ethyl acetate extract of the leaves of *C. latifolia* was most effective in inhibiting the bacteria, particularly against *S. aureus* and *S. epidermidis*. Further study is needed to explore higher concentrations or alternative extraction method to enhance the antibacterial activity.

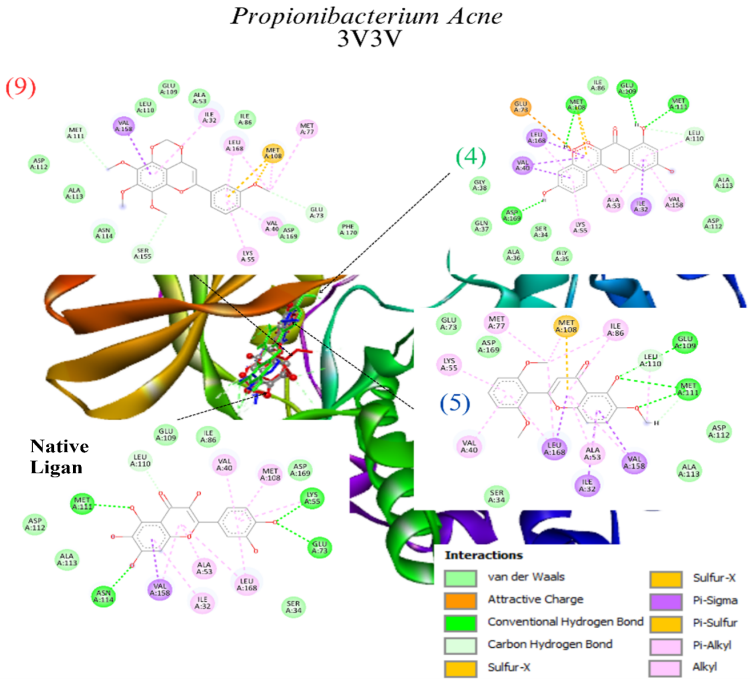


Fig. 5: Visualization result of *Propionibacterium acne* with 3 best substances. 2-(3,4-dihydroxyphenyl)-3,5,6,7-tetrahydroxy-chromen-4-one (Native ligand);tangeritin **(9)**; 2-(2,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-chromen-4-one **(4)**; 2-(2,6-dimethoxyphenyl)-5,6-dimethoxy-4H-chromen-4-one **(5)**

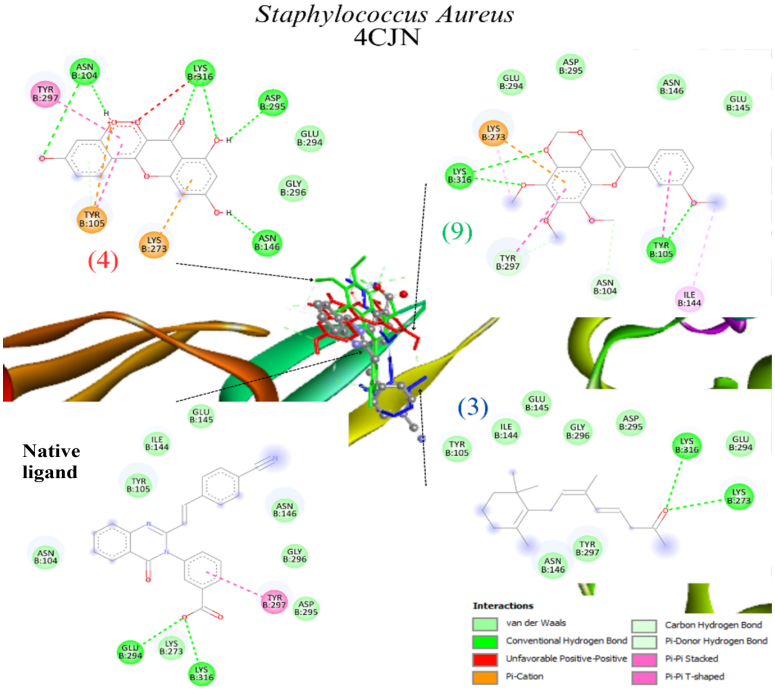


Fig. 6: Visualization result *Staphylococcus aureus* with three best compounds. *3*-[2-[(E)-2-(4-cyanophenyl)ethenyl]-4-oxidanylidene-quinazolin-3-yl]benzoic acid (Native ligand); 2-(2,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-chromen-4-one **(4)**; Tangeritin **(9)**;13-apo-beta-carotenone **(3)**

The appropriateness of the solvent in extracting natural materials dramatically affects the secondary metabolite profile of the extract (Hasnat *et al*. 2023). Ethyl acetate solvent is one of the solvents with a polarity index in the semipolar category. The semipolar characteristic of ethyl acetate can attract compounds that are also semipolar

Table 5: Free energy of binding score, inhibitory constanta and hydrogen interaction between substance and target protein

Sr. No	*Propionibacterium acne* (PDB ID: 3V3V)			*Staphylococcus aureus* (PDB ID: 4CJN)			*Staphylococcus epidermidis* (PDB ID: 3KP2)		
	FEB (kcal/mol)	IC	H-BOND	FEB (kcal/mol)	IC (μM)	H-BOND	FEB (kcal/mol)	IC (μM)	H-BOND
NL	-8.18	1.00 μM	**LYS55, MET111, ASN114, GLU73, LEU110**	-7.64	2.51	**LYS316, GLU294**	-3.67	2.03 m*M*	**LYS74**
1	-8.23	934.57 n*M*	-	-5.98	41.35	LYS273	-1.63	64.01 m*M*	**LYS74**
2	-7.78	1.98 μM	-	-5.66	71.33	**LYS316**	2.62E+05	-	-
3	-7.66	2.43 μM	**LYS55**	-5.9	47.21	LYS273, **LYS316**	-2.44	16.21 m*M*	**LYS74**
4	-8.43	665.15 n*M*	**MET111,** MET108, ASP169, GLU109, **LEU110**	-6.96	7.94	ASN104, **LYS316,** ASN146, ASP295, TYR105	-3.01	6.20 m*M*	ARG70, ASN64, ARG70
5	-7.93	1.54 μM	**MET111,** GLU109, **LEU110**	-5.54	87.37	TYR105, ASP275, ASP295, ILE144, LYS273	-2.39	17.85 m*M*	ARG70, ALA67, **LYS74**
6	-7.49	3.23 μM	**MET111,** GLU109, SER34, ASP169, **GLU73**	-5.27	136.19	ASN104, TYR105, ASN146	-1.98	35.45 m*M*	ASN64
7	24.76	-	**MET111,** ASP112	-4.32	685.40	**LYS316,** ILE144	1.17E+06	-	-
8	-7.11	6.12 μM	**MET111, ASN114,** ALA113, **GLU73,** ILE32	-5.27	137.32	TYR105, LYS273, ASN104, **GLU294,** ASP295	-1.11	152.88 m*M*	ALA67, ALA66, **LYS74**
9	-8.45	644.68 n*M*	**GLU73,** SER155, **MET111**	-6.51	16.81	TYR105, **LYS316,** ASN104, TYR297, LYS273	-1.95	36.97 m*M*	ASN64
10	-7.05	6.81 μM	SER34, ASP169, **GLU73**	-5.47	98.01	LYS273	-1.71	55.99 m*M*	ARG70, ASN64, **LYS74**

Description: Bold showed H-Bond which resemble native ligand, S: Substance, FEB: *Free Energy of Binding*, IC: inhibition constant, NL: Native Ligand

through the principle of like dissolves like. Some information has been discussed that semipolar compounds are thought to have a strong ability to inhibit bacterial activity (Wan 2017; Nur *et al*. 2022). Molecularly, semipolar compounds allow faster penetration into the bacterial cell to damage the bacterial cell membranes and the bacterial fatty acid synthesis (Islam *et al*. 2018). The antibacterial ability is shown by the ethyl acetate extract of *C. latifolia* leaves, so in this study, an LC-MS/MS analysis was carried out to identify compounds that could be responsible for their antibacterial activity (Nur *et al*. 2023a, b).

Liquid Chromatography-Mass Spectrometry (LC-MS/MS) is an analytical method that combines the high separation ability of liquid chromatography with precise detection ability of mass spectrometry. Each peak in the LC chromatogram represents a compound. The prominent peaks are subjected to MS analysis for the determination of their molecular weights and fragmentation pattern, such that each separated compound in the LC can be identified from its Mass spectrum data (Kaufmann 2012; Mangurana *et al*. 2019).

The LC-MS/MS analysis of the leaf extract resulted in several chromatographic peaks at various retention time. The chromatogram showed that 10 compounds were prominent in the leaf extract of *C. latifolia*. The MS spectrum of each compound was analyzed and compared with PubChem data base resulting in the identification of the compounds as (9-cis)-retinal (1), 10'-apo-beta-carotenal **(2),** 13-apo-beta-carotenone **(3)**, 2-(2,4-Dihydroxyphenyl)-3,5,7-trihydroxy-4H-chromen-4-one **(4)**, 2-(2,6-dimethoxyphenyl)-5,6-dimethoxy-4H-chromen-4-one **(5)**, 2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-3,4-dihydro-2H-1-benzopyran-4-one **(6)**, diadinoxanthin **(7)**, nobiletin **(8)**, tangeritin **(9)** and tetramethylscutellarein **(10)**. The compounds belong to the flavonoid and terpenoid groups of secondary metabolites and these compounds may likely responsible for the observed anti-bacterial activity of the ethyl acetate extract of *C. latifolia* leaves.

In this study, molecular docking analysis was carried out to evaluate the bioactivity of the compounds in their interactions with bacterial target proteins. In the analysis

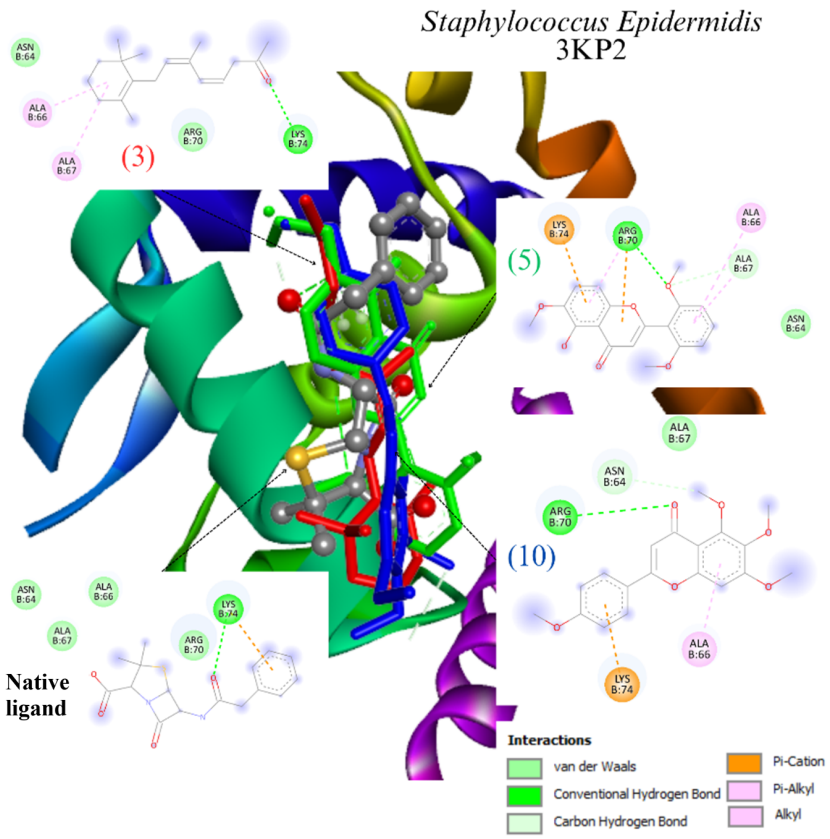


Fig. 7: Visualization result of *Staphylococcus epidermidis* with three best substance (2S,5R,6R)-3,3-dimethyl-7-oxo-6-(2-phenylethanoylamino)-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid (Native ligand);13-apo-beta-carotenone (3);2-(2,6-dimethoxyphenyl)-5,6-dimethoxy-4H-chromen-4-one **(5)**; tetramethylscutellarein **(10)**

technique through the molecular docking approach, it is very important to prepare the test ligand to form a stable conformation with low potential energy (Agu *et al.* 2023). The structure was optimized using the semi-empirical quantum mechanics method using the AM1 (Austin Model 1) force field, a very accurate and fast technique for calculating atomic and molecular quantities (Ion *et al.* 2021).

In molecular docking analysis, assessment parameters such as the inhibition constant (Ki), hydrogen bonds and bond free energy need to be evaluated. The inhibition constant (Ki) represents the concentration at which the inhibitor ligand occupies 50% of the protein receptor sites (Prasetiawati *et al.* 2022). Hydrogen bonding is an important parameter that shows the pattern of the interaction between the ligands and the receptors (Akbar *et al.* 2022). The binding free energy reflects the stability of the ligand-protein complex, low free energy value indicate an energetically favourable and more stable ligand-protein complex (Tambunan and Alamudi 2010; Noviardi and Fachrurrazie 2015). A high negative binding free energy and low Ki score indicate a spontaneous and stable interaction between the ligand and the target protein (Morris *et al.* 1998). The results from this study indicate that the test ligands formed a stable complex with the target proteins.

The values obtained for the binding free energy, inhibition constant and hydrogen bonding interaction for the test ligands were similar to that of the native ligand. Subsequently, three compounds with the best docking score with each of the target proteins were selected for the visualization study. These compounds were Tangeritin **(9)**, 2-(2,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-chromen-4-one **(4)** and 2-(2,6-dimethoxyphenyl)-5,6-dimethoxy-4H-chromen-4-one **(5)** for *P. acnes* (PDB ID: 3V3V), 2-(2,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-chromen-4-one **(4)**, tangeritin **(9)** and 13-apo-beta-carotenone **(3)** for *S. aureus* (PDB ID: 4CJN) (Fig. 6) and 13-apo-beta-carotenone **(3)**, 2-(2,6-dimethoxyphenyl)-5,6-dimethoxy-4H-chromen-4-one **(5)** and Tetramethylscutellarein **(10)** for *S. epidermidis* (PDB ID: 3KP2).

In terms of their binding free energy, inhibition constant and hydrogen bond interaction, 7 out of the 10 compounds tested showed better and more stable interaction with *P. acnes* (PDB ID: 3V3V) than the native ligand, 6 compounds showed better and more stable interaction with *S. aureus* (PDB ID: 4CJN) than the native ligand and 5 compounds showed better and more stable interaction with *S. epidermidis* (PDB ID: 3KP2) than the native ligand.

The ability of each compound to inhibit the growth of the bacteria is initiated by hydrogen and van der Waals interactions between the binding sites of the compound and key amino acids of the target proteins. As shown in Table 5, the key amino acid residues of each protein that interacted with the binding sites of the ligands were Met 111 and Glu109 for *P. acnes*, Lys316 and Gly294 for *S. aureus* and Arg70 for *S. epidermidis*.

Compounds 13-apo-beta-carotene **(3)**, 2-(2,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-chromen-4-one **(4)**, 2-(2,6-dimethoxyphenyl)-5,6-dimethoxy-4H-chromen-4-one **(5)**, tangeritin **(9)** and tetramethylscutellarein **(10)** are generally secondary metabolites from the phenolic/polyphenol group. The chemical interaction between the phenolic/polyphenol compounds and the bacterial target protein leads to the disruption of the bacterial cells, reduces the stability of the bacterial cell membranes and induces reactive oxygen species (ROS) production that can inhibit bacterial protein synthesis and transcription (Davidova *et al.* 2024).

It can therefore be stated that compounds 3, 4, 5, 9 and 10 from *Curculigo latifolia* are best candidate for development as antibacterial agents.

Conclusion

The results of the present study have shown that the ethyl acetate extracts of the leaves, stems and roots of *C. latifolia* possess the potential as antibacterial agents against *P. acnes, S. aureus* and *S. epidermidis*, although the effectiveness is less as compared to the reference antibiotic clindamycin. The stem and leaf extracts were more effective than the root extract. LC-MS/MS analysis identified ten compounds belonging to the flavonoid and terpenoid group of secondary metabolites in the ethyl acetate leaf extract of *C. latifolia*. Molecular docking study showed that these compounds displayed a spontaneous and stable interaction with the bacterial protein targets which include *P. acnes* (PDB ID: 3V3V), *S. aureus* (PDB ID: 4CJN) and *S. epidermidis* (PDB ID: 3KP2) with compounds 13-apo-beta-carotene **(3)**, 2-(2,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-chromen-4-one **(4)**, 2-(2,6-dimethoxyphenyl)-5,6-dimethoxy-4H-chromen-4-one **(5)**, tangeritin **(9)** and tetramethylscutellarein **(10)** showing better docking score than the native ligands of the target proteins. These compounds could therefore be potential candidates for development as antibacterial agents for the treatment of acne.

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Author's Contribution

BE, RS, RRS and SN: Conseptualization and designed research. RS and SN: Methodology, wrote the manuscript and analysis data. RRS and BE: Data interpretation, manuscript review and validation.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Data Availability

The data will be available on a fair request to the correspondence author.

Ethic Approval

This article does not contain any studies involving human or animal subjects.

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