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Research Article

In Vitro and In Silico Study of Antibacterial Activity of Sirih Merah Leaf Extract (*Piper crocatum* Ruiz & Pav.) Against *Streptococcus mutans*

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Abstract
Sirih merah leaf (*Piper crocatum Ruiz & Pav*) is a natural material that has the potential to be used as a natural antibacterial agent in treating dental caries caused by *Streptococcus mutans* because it is known to contain secondary metabolite compounds that have antibacterial properties that can inhibit the activity of glucosyltransferase and glucan-binding protein (Gbp) enzymes produced by S. mutans. This study was conducted to determine the antibacterial activity of Sirih merah leaf extract against S. mutans using the Kirby-Bauer method and to predict the potential molecular activity of antibacterial compounds contained in Sirih merah leaves in inhibiting the growth of S. mutans bacteria in silico using the molecular docking method. Antibacterial activity testing of Sirih merah leaf extract fractions obtained from methanol extract showed that fractions 7 and 10 are active fractions because they can inhibit the growth of *S. mutans* at the highest concentration of 10% with a strong inhibition zone category. The active fractions were then analyzed by the LC-HRMS method to obtain active compounds. The results of the in silico molecular docking test showed that the active compounds of Sirih merah leaves have the potential to interact with the GtfB and GbpC enzymes of *S. mutans*, where the best binding energy in the interaction of cafedrine compounds is -8.75 kcal/mol and -9.27 kcal/mol. The docking results were validated through a molecular docking process using native ligands with RMSD values of 1,861 Å and 3,170 Å.

Keywords: Antibacterial, glucan-binding protein, glucosyltransferase, sirih merah leaf, *Streptococcus mutans*

1. INTRODUCTION

The World Health Organization (WHO) Global Oral Health Status Report (2022) estimates that oral diseases affect nearly 3.5 billion people worldwide with dental caries being the most common[1]. Dental caries is localized damage to the teeth caused by the process of bacterial fermentation of carbohydrates derived from food debris contained in the oral cavity. The cause of dental caries is due to the synergistic interaction of bacteria such as *Streptococcus mutans* (*S. mutans*) which form a biofilm on the tooth surface[2].

Currently, many synthetic drugs have been developed that are used as treatments for dental caries. However, the continuous use of synthetic drugs is feared to have a negative impact on the human body, such as the occurrence of resistance [3]. To prevent this, it is necessary to use traditional medicines that have fewer side effects. One of the natural ingredients that can be used as an alternative to traditional medicines in treating dental caries caused by S. mutans bacteria is Sirih merah leaf [4]. Sirih merah leaf (*Piper crocatum Ruiz & Pav*) has the potential to be used as a natural antibacterial agent because it is known to contain secondary metabolite compounds that have antibacterial properties. These compounds include flavonoids, alkaloids, tannins, polyphenolic compounds, and essential oils. Essential compounds isolated from red betel leaves are terpenoid compounds, namely sabinen and mirsen, and have the potential as anti-bacterial *S. mutans* [5]. Research on

fraction 2 of Sirih merah leaf and insilico test study showed that Nootkatone compounds have the potential to interact with *Enterococcus faecalis* bacteria using the MurA enzyme which provides the best binding energy of -7.09 kcal/mol [6].

S. mutans bacteria are known to produce the enzyme glucosyltransferase (Gtf) [7]. Research by Erviana et al. (2011) [8] proved that essential oil compounds isolated from Sirih merah leaf extract can inhibit the growth of *S. mutans* bacteria by inhibiting the activity of glucosyltransferase enzymes produced by *S. mutans*. There are three types of glucosyltransferase enzymes that synthesise glucan polymers from sucrose, namely GtfB, GtfC, and GtfD6. However, the GtfB enzyme is an essential component of plaque biofilm that facilitates cell adhesion and accumulation of stable biofilm mediated by glucan-binding protein (Gbp) [9].

S. mutans also produces a nonenzymatic glucan-binding protein, glucan-binding protein (GBP). Gbp interacts with glucan synthesized by the enzyme glucosyltransferase (Gtf) from sucrose to form the extracellular matrix of the biofilm, which helps the bacteria colonize the tooth surface. The interaction between Gbp and glucan from Gtf enzymes is vital in biofilm formation and stability, which affects the pathogenicity of *S. mutans* [10]. One type of Gbp, GbpC, is an important component in biofilm formation. This is because GbpC is known to mediate the adhesion process, both independently and dependent on sucrose [11].

Based on the information above, research on the isolation of Sirih merah extract and its antibacterial test against *S. Mutans* bacteria as well as studies on the molecular docking mechanism of its active compounds have not been reported.

This research was conducted in vitro through the process of isolating active compounds contained in Sirih merah leaf extract and testing the antibacterial activity of these active compounds against *S. Mutans* bacteria. Next, in silico analysis was conducted on the suspected compound structure using molecular the docking method to predict the potential molecular activity of antibacterial compounds contained in Sirih merah leaves in inhibiting the growth of *S. mutans* bacteria.

2. RESEARCH METHODS

Instruments and Materials

Sirih merah leaves (*Piper crocatum Ruiz & Pav*.) were obtained from the Cibeunying Kaler area, Bandung City. Plant determination was carried out at the Biology Laboratory, Faculty of Mathematics and Natural Sciences, Padjadjaran University, Sumedang. Distilled organic solvents: ethyl acetate, methanol, and n-hexane. Rotary vacuum evaporator (Stuart). Chloroform, NH_4OH, H_2SO_4 2M, Dragendorff, Mayer, Wegner reagents, 30% methanol, 1% $FeCl_3$, 30% ethanol, Liebermann-Burchard reagent, and distilled water were used for phytochemical screening. Merck Silica G60 (0.063-0.200 mm) and Merck Silica G60 (0.2-0.5 mm). TLC aluminium sheets silica gel 60 F254 20x20 cm, UV lamp Vilber Lourmat VL-8.LC wavelength 254 and 366 nm. *Streptococcus mutans* ATCC 25175, Brain Hinton Infusion Broth (BHI-B), Mueller Hinton Agar (MHA) medium, chlorhexidine, McFarland standard as well as UV-Vis Spectrophotometer instrument (Shimadzu 1240). Liquid Chromatography-High Resolution Mass Spectroscopy (LC-HRMS) from Thermoscientific Orbitrap Exploris 120 with ZORBAX EclipsePlus C18RRHD 2.1 mm x 100 mm x 1.8 µm column from Agilent, formic acid 0.1% and acetonitrile as mobile phase. For in silico testing, hardware and software were used such as an ASUS Vivobook AMD Ryzen 3 4300U with Radeon Graphics 2.70 GHz with Windows 11 operating system, 64-bit operating system, x64-based processor, Research Collaboratory for Structural Bioinformatics (RCSB), PubChem (NCBI), Chemdraw 3D, Biovia Discovery Studio Visualize (BIOVIA), Autodock 4.0 and Amber.

Extraction of Sirih merah Leaf (*P. crocatum*)

A total of ± 2 kg of Sirih merah leaf (*P. crocatum*) was cleaned from impurities, then dried in an open room, and mashed. Sirih merah leaves were macerated using methanol solvent for 3x24 hours. After filtering, a rotary evaporator was used to evaporate the filtrate at a temperature of ± 40ºC until a thick extract was achieved.

Fractionation of Sirih merah Leaf (*P. crocatum*) Methanol Extract by Gravity Column Chromatography (GCG) Method

Silica gel 60 (0.063-0.200 mm) was inserted into the chromatography column. A total of 10 grams of sample was added with acetone until completely dissolved and impregnated into silica gel 60 (0.2-0.5 mm) with the ratio of sample and silica gel 60 (0.2-0.5 mm) (1:1). The impregnated sample was then inserted into a chromatography column with a diameter of 5.5 cm just above the silica gel 60 (0.063-0.200 mm) that had been monolayered with 100% n-hexane, then eluted using 100% n-hexane solvent with a variation of n-hexane: ethyl acetate eluent that graded its polarity until it ended with elution using 100% methanol. The fractions obtained were subjected to KLT control using n-hexane: ethyl acetate (6:4) eluent and the resulting stains were observed under UV light λ 254 and 366 nm.

Antibacterial Activity Test against S. mutans Kirby-Bauer Method

Planting and Determination of the Number of Bacteria

Streptococcus mutans bacteria as much as 3 mL were implanted into Brain Hinton Infusion Broth (BHI-B) media and then incubated at 37 ºC for 48 hours. Using a UV-Vis Spectrophotometer, the absorbance at λ 600 nm was measured after 1 mL of the bacterial culture in the liquid media was transferred into a cuvette. The absorbance value was entered into the equation $A_1V_1 = A_2V_2$ ($A_2 = 0.132$, 0.5 McFarland value). If the absorbance value is > 0.132 then media is added. If the absorbance value is < 0.132 then add bacteria [12].

Media Preparation

Twenty milliliters of Mueller Hinton Agar medium were placed in a petri dish at 40-50 ºC, shaken to homogenize the mixture, and then left to stand at room temperature until it hardened and was ready to use.

Testing of Samples, Negative Controls, and Positive Controls

Mueller Hinton Agar medium was smeared with 100 µL of bacterial suspension using a spreader bar. Samples of betel leaf fraction, methanol as negative control, and chlorhexidine as positive control were dripped on a paper disk as much as 20 µL. Each paper disk containing the test solution was inserted into solid media that had been inoculated with S. mutans bacteria. Incubated at 37 ºC for 48 hours. Observed that the diameter of the inhibition zone was marked by the formation of a clear zone area around the paper disk, then measured using a caliper.

LC-HRMS Analysis

LC-HRMS analysis was used to identify the suspected active compounds contained in the fractionated methanol extract of Sirih merah leaf. The analysis was carried out using a ZORBAX EclipsePlus C18RRHD 2.1 mm x 100 mm x 1.8 µm C18 column from Agilent as the stationary phase. The mobile phase consisted of formic acid 0.1% and acetonitrile using a gradient system. The injection volume was 10 µL with a running time of 43 minutes and a mass range of 50-750 m/z. The mass spectra obtained were then corrected against solvent blanks and data processing software filters in the natural products library used in testing instruments at the Advanced Chemistry Advanced Characterization Laboratory of the National Research and Innovation Agency (BRIN).

Molecular Docking in Silico Assay

The 3D structures of GtfB enzyme and GbpC protein used in this study were obtained from Protein Data Bank (PDB) with ID: 8FG8 (GtfB) (https://www.rcsb.org/structure/8FG8) and 6CAM (GbpC) https://www.rcsb.org/structure/6CAM). These enzymes and proteins act as receptors, so it is necessary to perform structural optimization to ensure that the enzyme or protein model used in the simulation is representative and ready to interact with the ligand [13]. GtfB enzyme and GbpC protein molecules were cleaned from water molecules, ions, or irrelevant ligands so as not to hinder the molecular docking process[14] using BIOVIA Discovery Studio software. After that, hydrogen atoms were added to ensure accurate hydrogen bonding interactions [13].

The 2D and 3D structures of cafedrine (CID 7174), ethyl [(5-amino 1H-1,2,4-triazol-3-yl)sulfanyl]acetate (CID 1221706), 5,8-Dimethyl-1-Tetralone (CID 99334), and 2-(1,3-Dimethyl-2,6-dioxo-1,2,3, 6-tetrahydro-7H-purin-7-yl)-N-(2-methylcyclohexyl)acetamide (CID 319200315) as an active compound found in Sirih merah leaf acts as a ligand obtained from PubChem with SDF format. Then, energy minimization was performed using ChemBio 3D Ultra 12.0 software to optimize the ligand conformation[15]. Meanwhile, the compound 2-[(2,4,5-Trihydroxyphenyl) methylidene]-1-benzofuran-3-one) (CID 153126818) acts as a native ligand found in the GtfB enzyme, the compound β-D-glucopyranose (CID 64689) acts as a native ligand found in the GbpC protein, and chlorhexidine (CID 9552079) acts as a positive control used in the in vitro test. The molecular docking process was carried out using AutoDock Tools software (https://autodock.scripps.edu/), then the results of receptor and ligand interactions were visualized using BIOVIA Discovery Studio (https://www.3ds.com/products/biovia/discovery-studio).

3. RESULTS AND DISCUSSION

Extraction of Sirih merah Leaf (*P. crocatum*)

A total of 1.95 kg of dry and delicate Sirih merah leaf samples were extracted using the solid-liquid maceration method. The maceration process was carried out by placing the Sirih merah leaf powder sample a tightly closed inert container at room temperature, then soaking it in using 20 L of methanol solvent 3 times 24 hours. At the extraction stage, methanol as a solvent will be able to separate the components of the substance from the mixture based on the principle of like dissolve like. Polar compounds will dissolve in polar solvents, while nonpolar compounds will also dissolve in nonpolar solvents [16]. The methanol solvent was then separated, then concentrated using a vacuum rotary evaporator. The vacuum contained in the rotary evaporator can reduce the pressure in the system, allowing the methanol solvent to boil at a lower temperature than its boiling

point at room pressure [17]. From this evaporation process, a thick extract of Sirih merah leaves as much as 275.72 g was obtained.

Fractionation of Sirih merah Leaf Methanol Extract (*P. crocatum*) and Antibacterial Activity Test against S. mutans Kirby-Bauer Method.

The concentrated methanol extract of Sirih merah leaves was then fractionated using gravity column chromatography. The elution process was carried out in a gradient manner with the aim of obtaining fraction groups according to the level of polarity[18]. From this fractionation stage, 13 fractions were obtained which were then continued with the anti-bacterial activity test against S. Mutans using the Kirby-Bauer method.

The principle of the Kirby-Bauer method is to measure the diameter of the clear zone around the paper disc[19] which aims to assess the ability of the active compounds in Sirih merah leaf extract to inhibit the growth of *S. mutans* bacteria.

Samples of Sirih merah leaf extract (13 fractions) were made into 4 concentration variations, namely 4, 6, 8, and 10%. Chlorhexidine 2% was used as a positive control because chlorhexidine is a broad-spectrum antiseptic drug that is bactericidal against bacteria and is a commonly used standard in the treatment of periodontal disease [20]. The negative control used was methanol which is the sample solvent. The sample solution, positive control, and negative control were dripped on a paper disc as much as 20 µL.

Mueller-Hinton Agar (MHA) media in a solidified petri dish was inoculated with 100 µL of S. mutans bacterial suspension and then leveled using a spreader bar. The solid media that had been inoculated with S. mutans bacteria were then filled with paper discs that contained the test solution, positive control, and negative control. The samples were incubated in an incubator at 37 ºC for 48 hours. The zone of inhibition formed was measured in diameter using a caliper. The resulting zone of inhibition was categorized based on Davis & Stout (1971) [21]. The results of the fractionation and anti-bacterial activity test can be seen in **Table 1**. It can be seen that fraction 7 and fraction 10 of Sirih merah leaf extract (*P. crocatum*) are active fractions because they can inhibit the growth of S. mutans bacteria at a concentration of 10% resulting in a strong inhibition zone category.

Table 1. Antibacterial activity test results of sirih merah leaf extract (*P. crocatum*) against *Streptococcus mutans*

Fraction	Fraction Weights (mg)	Diameters of Inhibition Zone					
		K (+)	K (-)	4%	6%	8%	10%
		(mm)					
1	47,8	19,8	0	0	0	0	0
2	80,8	19,85	0	0	0	0	0
3	245,5	23,9	0	6,8	6,8	6,8	8,2
4	342,4	22,4	0	6,8	6,85	7,2	8
5	324,1	19,0	0	0	0	0	0
6	852,7	17,7	0	6,6	6,7	6,8	7,2
7	2.213,4	19,5	0	6,2	6,5	7,2	11,6
8	472,4	17,4	0	6,6	6,8	7,1	8,1
9	293,9	18,5	0	6,5	6,9	7,5	7,8
10	38,5	18,5	0	6,6	6,9	7,65	15,1
11	26,8	19,45	0	0	0	0	0
12	50,5	31,1	0	6,0	6,1	6,7	7,2
13	1.326,2	33,1	0	0	0	0	0

LC-HRMS analysis

The active fractions obtained based on the results of the In Vitro antibacterial activity test, namely fraction 7 and fraction 10 were then analyzed by LCHR-MS in order to obtain the alleged compounds contained in the two fractions. The analysis results for fractions 2 and 10 can be seen in **Table 2** and **Table 3**, respectively.

Molecular Docking in Silico Test

In silico analysis was performed using molecular docking. This computational technique predicts the optimal orientation of a small molecule (ligand) when bound to a target protein (receptor) to form a stable complex[22]. Molecular docking is very important in drug design because it allows researchers to evaluate the interaction between a drug candidate

and its biological target in silico, i.e. through computer simulation [23]. In this assay, the suspected active compounds obtained based on the results of LCHR-MS analysis acted as ligands. Meanwhile, the targeted enzyme and protein, namely the GtfB enzyme and GbpC protein, act as receptors.

To determine the ability of active compounds of Sirih merah leaf extract to inhibit protein activity, the bond energy value of the calculation results was selected. The lower the bond energy value produced, the more stable and stronger the complex formed between the receptor and ligand [24,25]. The analysis results shown in **Table 4** represent the interactions that occur between protein-ligand expressed in energy (kcal/mol). The energy value shows the strength of the interaction formed between each active compound contained in the Sirih merah leaf extract (*P. crocatum*) with the GtfB enzyme and the GbpC protein.

Table 2. Results of LC-HRMS analysis of alleged compounds in fraction 7 of sirih merah leaf extract (*P. crocatum*)

No	Compound Name	Formula	Molecular Weights	(m/z)	RT (min)	Intensity
1	5,8-Dimethyl-1-Tetralone	$C_{12}H_{14}O$	174.10453	175.11177	38.25	4.9E+06
2	2-(1,3-Dimethyl 2,6-dioxo-1,2,3,6-tetrahydro-7H purin-7-yl)-N-(2-methylcyclohexyl) acetamide	$C_{16}H_{23}N_5O_3$	333.17888	334.18616	13.36	4.6E+06
3	(2E,4E)-Deca-2,4-dienal	$C_{10}H_{16}O$	152.12021	194.15401	0.73	7.2E+05
4	1-(1-Piperidinyl)- 3- {[(3aR,5aS,8aS,8b R)-2,2,7,7- tetramethyltetrahydro-3aH-bis[1,3] dioxolo[4,5 -b:4',5'-d]pyran-5- yl]methoxy}-2-propanol	$C_{20}H_{35}NO_7$	401.24147	402.24875	20.93	7.0E+06
5	3,4-Dihydroxyphenylglycol	$C_8H_{10}O_4$	170.05799	171.06525	0.73	6.0E+06
6	Acrolein	C_3H_4O	56.02627	74.06008	38.03	1.8E+07
7	Butyl acrylate	$C_7H_{12}O_2$	128.08379	111.08051	38.21	5.3E+07
8	Guaiacol	$C_7H_8O_2$	124.05252	125.05980	38.21	1.4E+08
9	N-(2-{8-[2-(1H-Imidazol-4-yl)ethyl]-9-oxo2,8-diazaspiro[5.5]undec-2-yl}-2-oxoethyl)acetamide	$C_{18}H_{27}N_5O_3$	361.21019	362.21747	17.75	1.6E+07

Table 3. Results of LC-HRMS analysis of alleged compounds in fraction 10 of sirih merah leaf extract (*P. crocatum*)

No	Compound Name	Formula	Molecular Weights	(m/z)	RT (min)	Intensity
1	5,8-Dimethyl-1-Tetralone	$C_{12}H_{14}O$	174.10453	175.11177	38.23	1.4E+06
2	Cafedrine	$C_{18}H_{23}N_5O_3$	357.17903	390.21243	38.06	8.2E+06
3	Ethyl [(5-amino-1H-1,2,4-triazol-3-yl)sulfanyl]acetate	$C_6H_{10}N_4O_2S$	202,05321	102,03388	38,23	4.8E+06
4	2-(1,3-Dimethyl 2,6-dioxo-1,2,3,6-tetrahydro-7H purin-7-yl)-N-(2-methylcyclohexyl) acetamide	$C_{16}H_{23}N_5O_3$	333.17888	334.18616	13.09	4.20E+07
5	(2E,4E)-Deca-2,4-dienal	$C_{10}H_{16}O$	152.12021	194.15401	0.73	8.0E+06
6	1-(1-Piperidinyl)- 3- {[(3aR,5aS,8aS,8b R)-2,2,7,7- tetramethyltetrahydro-3aH-bis[1,3]dioxolo[4,5 -b:4',5'-d]pyran-5-yl]methoxy}-2- propanol	$C_{20}H_{35}NO_7$	401.24147	402.24875	20.92	5.2E+06
7	3,4-Dihydroxyphenylglycol	$C_8H_{10}O_4$	170.05799	171.06525	0.73	4.7E+06
8	2,2'-{[2-({2-[2-Hydroxy-3-(2-methyl-1-aziridinyl)propyl]cyclopropyl}methoxy)-1,3-propanediyl]bis(oxy)}di(1,3-propanediol)	$C_{19}H_{37}NO_8$	407,25209	390,24881	38,06	8.2E+06
9	(E,E)-2,4-Heptadienal	$C_7H_{10}O$	110.07317	111.08044	38.21	2.9E+07
10	2-oxa-4-azatetracyclo[6.3.1 .1~6,10~.0~1,5~]tridecan-3-one	$C_{11}H_{15}NO_2$	193.11046	194.11774	0.74	8.0E+06
11	Pandangolide 1a	$C_{12}H_{20}O_5$	244,13135	227,12807	0,73	0.8E+06
12	Tetrahydrofurfuryl methacrylate	$C_9H_{14}O_3$	170,09440	153,09109	0,74	1.2E+05

Table 4. Molecular docking in silico test results of active compounds of sirih merah leaf (*P. crocatum*) with GtfB enzyme and GbpC protein against positive control

Protein-Ligand Interactions				
Compound	**GtfB (kkal/mol)**	**Ki**	**GbpC (kkal/mol)**	**Ki**
Cafedrine	-8.75	383.68 nM	-9.27	159.21 nM
Ethyl [(5-amino1H-1,2,4-triazol-3-yl)sulfanyl]acetate	-6.52	16.51 µM	-6.93	8.36 µM
2-(1,3-Dimethyl-2,6-dioxo-1,2,3,6-tetrahydro-7H purin-7-yl)-N-(2-methylcyclohexyl)acetamide	-6.94	13.18 µM	-4.19	329.51 µM
5,8-Dimethyl-1-Tetralone	-5.47	98.12 µM	-6.63	13.90 µM
(2E,4E)-Deca-2,4-dienal	-4.2	829.6 µM	-5.31	127.72 µM
1-(1-Piperidinyl)-3-{[(3aR,5aS,8aS,8b R)-2,2,7,7-tetramethyltetrahydro-3aH-bis[1,3]dioxolo[4,5 -b:4',5'-d]pyran-5-yl]methoxy}-2-propanol	-6.17	29.87 µM	-5.84	121.20 µM
3,4-Dihydroxyphenylglycol	-4.22	800.02 µM	-5.9	47.22 µM
Acrolein	-3.35	3.53 mM	-2.96	6.76 mM
Butyl acrylate	-3.64	2.15 mM	-4.58	439.63 µM
Guaiacol	-3.98	1.20 mM	-5.00	217.29 µM
N-(2-{8-[2-(1H-Imidazol-4-yl)ethyl]-9-oxo2,8-diazaspiro[5.5]undec-2-yl}-2-oxoethyl)acetamide	-6.39	18.29 µM	-6.41	27.46 µM
2,2'-{[2-({2-[2- Hydroxy-3-(2- methyl-1-aziridinyl)propyl]cyclopropyl}methoxy)-1,3-propanediyl]bis(oxy)}di(1,3-propanediol)	-2.98	6.51 mM	-6.50	17.27 µM
(E,E)-2,4-Heptadienal	-3.76	1.76 mM	-5.31	612.74 µM
2-oxa-4-azatetracyclo[6.3.1 .1~6,10~.0~1,5~]tridecan-3-one	-5.14	169.54 µM	-6.61	14.02 µM
Pandangolide 1a	-5.23	146.78 µM	-6.01	39.10 µM
Tetrahydrofurfuryl methacrylate	-4.11	969.48 µM	-5.62	75.76 µM
Chlorhexidine	-14.21	38.24 pM	-12.38	839.97 pM
2-[(2,4,5-Trihydroxyphenyl)methylidene]-1-benzofuran-3-one	-7.68	2.35 µM	-	-
β-D-glucopyranose	-	-	-5.59	79.75 µM

Table 4 shows that the presumptive compounds Cafedrine, Ethyl[(5-amino1H-1,2,4-triazol-3-yl)sulfanyl]acetate,2-(1,3-Dimethyl-2,6-dioxo-,2,3,6-tetrahydro-7Hpurin-7-yl)-N-(2-ethylcyclohexyl)acetamide and 5,8-Dimethyl-1-Tetralone provide relatively lower protein-ligand interaction energy values than other presumptive compounds.

The interaction between the cafedrine compound and the GtfB enzyme showed the lowest binding energy value compared to other active compounds, which amounted to -8.75 kcal/mol. The same phenomenon was also observed in the results of the interaction formed between cafedrine compounds and the GbpC protein, which showed a binding energy value of -9.27 kcal/mol. The low value of the bond energy formed indicates that cafedrine, an active compound contained in Sirih merah leaf extract (*P. crocatum*), has the potential as an antibacterial agent to inhibit Streptococcus mutans. **Figures 1** and **2** show the interactions formed between the receptor and ligand, which are in the form of hydrogen bonds shown in green and hydrophobic bonds in pink, purple, and orange. Presumptive compounds contained in the sample include cafedrine marked in red, ethyl[(5-amino1H-1,2,4-triazol-3-yl)sulfanyl]acetate in fuchsia, 2-(1,3-Dimethyl-2,6-dioxo-1,2,3,6-tetrahydro-7Hpurin-7-yl)-N-(2-ethylcyclohexyl)acetamide in yellow, and 5,8-Dimethyl-1-Tetralone in cloud blue. As a positive control, chlorhexidine was used which was marked in brown. Additionally 2-[(2,4,5-Trihydroxyphenyl)methylidene]-1-benzofuran-3-one and β-D-glucopyranose as native ligands which were marked in green. The hydrogen bond formed between the receptor and the ligand shows a weak interaction. This is due to the small number of hydrogen bonds formed between each active compound and the receptor. Stronger and more hydrogen bonds can reduce the bond energy value, which indicates the greater affinity of the ligand (active compound) with the receptor (target protein).

However, the amount of affinity between the ligand and the receptor is not only determined by the

number and strength of hydrogen bonds, but is also influenced by various other interactions formed at the binding site. The compounds cafedrine, ethyl[(5-amino1H-1,2,4-triazol-3-yl)sulfanyl]acetate, and 2-(1,3-Dimethyl-2,6-dioxo-1,2,3,6-tetrahydro-7Hpurin-7-yl)-N-(2-methylcyclohexyl)acetamide have only one hydrogen bond at Asp B:562 for GtfB shown in green. The same hydrogen bond was also found in chlorhexidine as a positive control. At the same time the interaction that occurs with the compound 5,8-Dimethyl-1-Tetralone shows the formation of one hydrogen bond at Gln B: 566 just like 2-[(2,4,5-Trihydroxyphenyl)methylidene]-1-benzofuran-3-one as a natural ligand found in the GtfB enzyme. The interaction formed between the compounds cafedrine, ethyl [(5-amino1H-1,2,4-triazol-3-yl)sulfanyl]acetate, and 2-(1,3-Dimethyl-2,6-dioxo-1,2,3,6-tetrahydro-7H purin-7-yl)-N-(2-methylcyclohexyl)acetamide with GbpC has one hydrogen bond shown in green at Ser A:347. Chlorhexidine as a positive control also showed the formation of one hydrogen bond at the same amino acid as the test compound. While β-D-glucopyranose as a natural ligand found in the GbpC protein shows hydrogen bonds at amino acids Ser A:347 and Ala A:453. Many hydrophobic bonds are formed between the active compound and other amino acid residues as shown in **Table 5**. Such interactions can occur on residues that are nonpolar or hydrophobic which usually occur in regions within enzymes or proteins that are less polar. Hydrophobic interactions are known to facilitate positioning of the ligand within the active site and reduce the total free energy of the system[26]. Some other theories suggest that hydrogen bonds frequently contribute to the stability of ligand-protein complexes and are often found on the active site of proteins [27].

Table 5. Receptor-amino acid interactions with GtfB and GbpC

Ligand	Receptor-Amino Acid Interactions			
	GtfB		GbpC	
	Hydrogen Bond	Hydrophobic Bond	Hydrogen Bond	Hydrophobic Bond
Cafedrine	Asp B:562	Ala B:452	Ser A:347	Phe A:452, Val A:410
Ethyl [(5-amino1H-1,2,4-triazol-3-yl)sulfanyl]acetate	Asp B:562	Ala B:452	Ser A:347	Ala A:453
2-(1,3-Dimethyl-2,6-dioxo-1,2,3,6-tetrahydro-7H purin-7-yl)-N-(2-methylcyclohexyl) acetamide	Asp B:562	Ala B:452, Phe B:881	Ser A:347	Ala A:453, Val A: 410
5,8-Dimethyl-1-Tetralone	Gln B:566	Ala B:452	Ser A:346	Phe A:452, Val A:410
Chlorhexidine	Asp B:562, Gln B:566	Phe B:881	Ser A:347	Ala A:453
2-[(2,4,5-Trihydroxyphenyl)methylidene]-1-benzofuran-3-one	Gln B:566	Ala B:452	-	-
β-D-glucopyranose	-	-	Ser A:347, Ala A:453	-

The binding affinity between the ligand and the receptor is influenced by the number of hydrogen bonds; the greater the number of hydrogen bonds, the stronger the interaction. Moreover, the amino acid residues involved in the binding show similarity to those interacting with chlorhexidine, which was used as a positive control—namely ASP B:562, Gln B:566, Ser A:347, and Ala A:453.

The growth of the *Streptococcus mutans* bacterium is efficiently inhibited by cafedrine compounds, according to the results of molecular docking. This can occur due to the ability of cafedrine to inhibit the activity of the GtfB enzyme and GbpC protein with the lowest binding energy value compared to other active compounds contained in Sirih merah leaf extract (*P. crocatum*) and native ligand compounds as positive controls, but not yet able to beat chlorhexidine.

The docking results were validated through a molecular docking process using native ligands, namely 2-[(2,4,5-trihydroxyphenyl)methylidene]-1-benzofuran-3-one for the GtfB enzyme and β-D-glucopyranose for the GbpC protein. Several researchers consider an RMSD value of up to 2 Å to be within an acceptable range [28].

The RMSD value obtained for the GbpC enzyme was 1.861 Å, indicating that the docking procedure was valid. In contrast, the RMSD value for the GtfB enzyme was 3.170 Å, suggesting that the docking method used is unreliable or invalid, as the RMSD exceeded the 2 Å threshold [28].

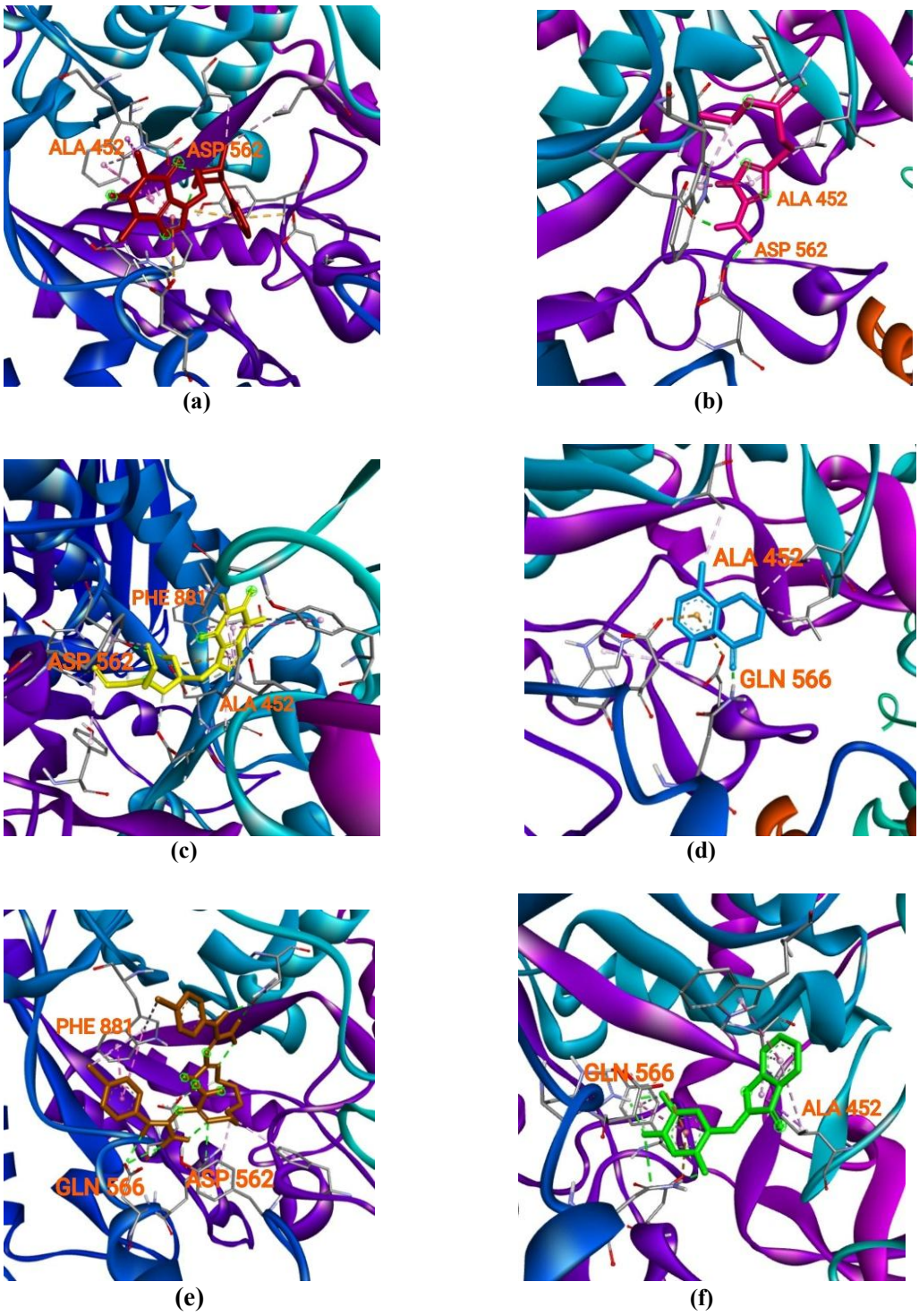

Figure 1. Visualization of interaction of active compounds with GtfB enzyme (a) Cafedrine (b) Ethyl [(5-amino1H-1,2,4-triazol-3-yl)sulfanyl]acetate (c) 2-(1,3-Dimethyl-2,6-dioxo-1,2,3, 6-tetrahydro-7H purin-7-yl)-N-(2-methylcyclohexyl)acetamide (d) 5,8-Dimethyl-1-Tetralone (e) Chlorhexidine (f) 2-[(2,4,5-Trihydroxyphenyl)methylidene]-1-benzofuran-3-one

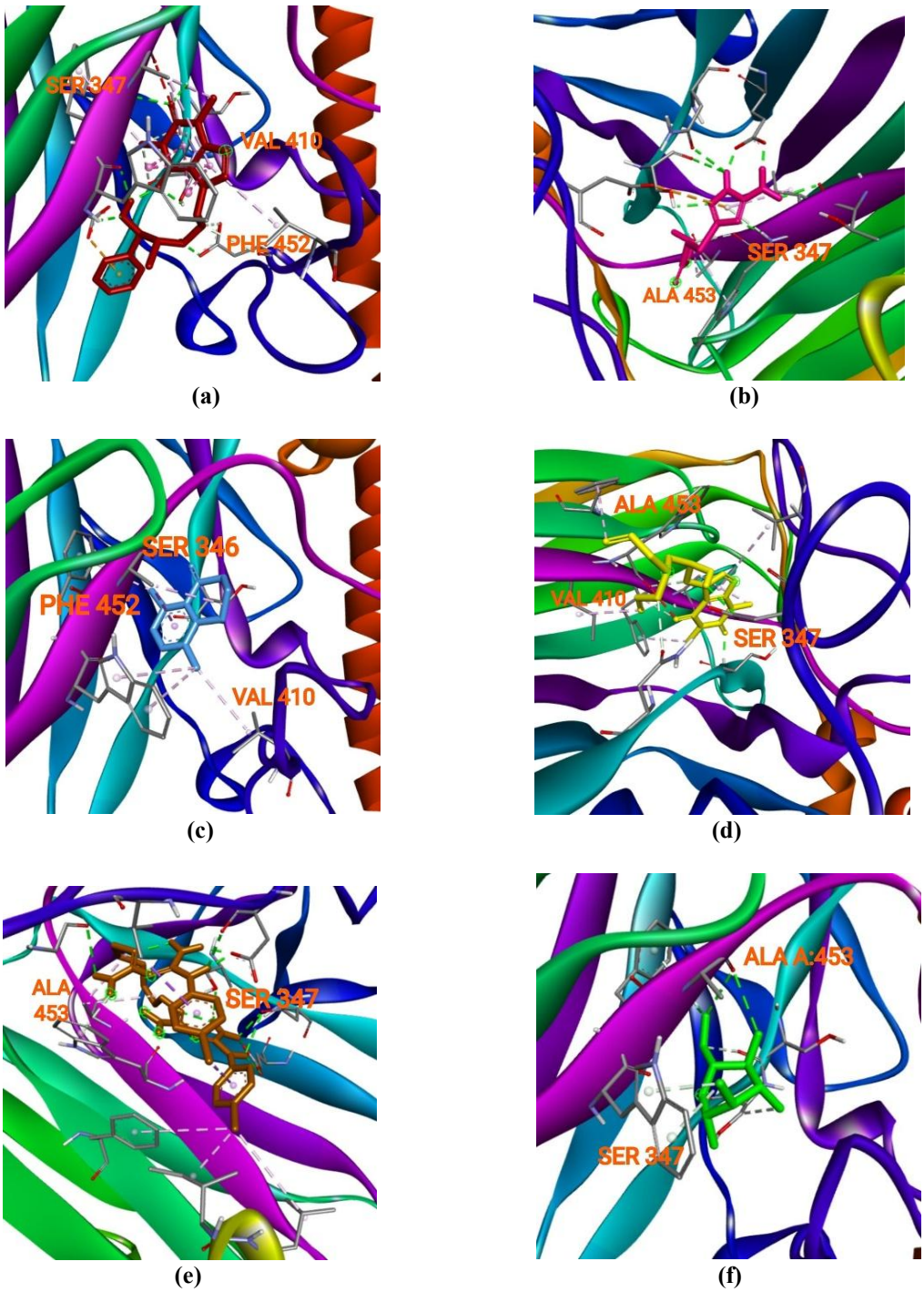

Figure 2. Visualization of interaction of active compounds with GbpC (a) Cafedrine (b) Ethyl [(5-amino1H-1,2,4-triazol-3-yl)sulfanyl]acetate (c) 5,8-Dimethyl-1-Tetralone (d) 2-(1, 3-Dimethyl-2,6-dioxo-1,2,3,6-tetrahydro-7H purin-7-yl)-N-(2-methylcyclohexyl)acetamide (e) Chlorhexidine (f)) β-D-glucopyranose

4. CONCLUSIONS

The results of the In Vitro antibacterial activity test of Sirih merah extract fractions against S. mutans showed that fraction 7 and fraction 10 were active fractions because they could inhibit bacterial growth at the highest concentration of 10% with a strong inhibition zone category. In test results from molecular docking showed that the active compounds of Sirih merah leaf have the potential to interact with the GtfB and GbpC enzymes of S. mutans, with the best binding energies found for the cafedrine compounds at -8.75 kcal/mol and -9.27 kcal/mol. For further research, the GtfB enzyme needs to be docked with other native ligand compounds. The active compound analysed by LC-HRMS needs to be isolated as a target compound for toxicity testing as well as in vivo testing.

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