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Antibacterial Activity of Legundi Leaf (*Vitex trifolia*) Essential Oil Using *in-vitro* and *in-silico* Methods

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ABSTRACT

Staphylococcus aureus causes many severe diseases, such as meningitis, lung infection, and hematogenous osteomyelitis. The prolonged use of antibiotic drugs leads to antimicrobial resistance (AMR), decreasing their effectiveness against *S. aureus*. Therefore, novel drugs isolated from natural products have been the focus of many researchers to solve that problem. The essential oils isolated from *Vitex trifolia* leaf have many pharmacological activities, including antibacterial and antifungal activities. In this research, essential oil was isolated by distillation, followed by GC-MS analysis. The antibacterial activity of the essential oil was conducted using a disc diffusion test (Kirby-Bauer) and by molecular docking study. The essential oil yield from distillation was 0.13%, containing major compounds such as sabinene, eucalyptol, terpinen-4-ol, α-terpineol, limonene, and caryophyllene. The essential oil gave intermediate inhibition against *S. aureus* with a 6.91 ± 0.03 mm inhibition zone in 20 mg/mL of the sample. Using molecular docking study, caryophyllene and α-terpineol were the key compounds for inhibiting the active site of tyrosyl-tRNA synthetase, sortase A, and dihydrofolate reductase that are responsible for the *S. aureus* growth and development.

Key words: Vitex-trifolia, oil, antibacterial, Staphylococcus aureus; docking-study

INTRODUCTION

The bacterium of *Staphylococcus aureus* is the primary pathogen in humans and normal flora on their skins and mucous membranes [1]. Its infections vary from food poisoning to mild and severe skin infections that can threaten human lives. The spread and development of *S. aureus* cause severe endocarditis, hematogenous osteomyelitis, meningitis, and lung infection [2,3]. Antibiotics are needed if bacteria develop rapidly or produce toxic compounds. Antibiotics have several mechanisms to inhibit bacterial growth: (i) destroying cell walls, (ii) inhibiting the reproduction processes, and (iii) stopping the protein production from bacteria [4,5]. Effective antibiotics are the ones that can occupy the bacteria's binding site in the infected tissue. However, the high usage of antibiotic drugs leads to an increase in antimicrobial resistance (AMR) [6–8]. The AMR can decrease the efficacy of antibacterial, antivirus, and antifungal drugs. It causes prolonged disease, expensive medical costs, and increased death. Therefore, finding new sources of antibacterial agent from nature become an alternative [9,10].

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Legundi plant (*Vitex trifolia*) is a shrub that grows well in tropical areas in South-East Asia. It is distributed in Indonesia, Philippine, Vietnam, Thailand and Papua New Guinea [11]. Every part of this plant has several bioactivities due to its secondary metabolites. The part of leaves, flower, stembark, and roots was reported to have antibacterial [12–16] and antifungal [17,18] activities. This part contains essential oils with the main compounds of monoterpene such as 1,8-cineole, terpinyl acetate, sabinene, α-pinene and sesquiterpene such as caryophyllene, bicyclogermacrene, and spathulenol [14,15,19,20].

In this paper, will be reported our recent isolation of Indonesian variant of *Vitex trifolia* essential oil, evaluation their antibacterial *in-vitro* and *in-silico*. The *in-vitro* strategy was undergone following Kirby-Bauer antibacterial [21,22] test toward *S. aureus* growth using disc diffusion. Meanwhile *in-silico* strategy was applied using a molecular docking based-computational [23]. This technique visualizes the interactions between side active compounds of ligand derived from molecule isolated in the *V. trifolia* essential oil and protein. Three type proteins correlate to bacterial growth inhibitions applied such as tyrosyl-tRNA synthetase (PDB ID: 1JIJ) [24]. This protein plays an important role in the formation of protein. Then, protein of sortase A (PDB ID: 2MLM) [25,26]. This protein has a role in sorting and connecting protein to peptidoglycan in the cell wall of gram-positive bacteria. Last, protein of dihydrofolate reductase (PDB ID: 3FRA) [27,28]. It has a crucial precursor in DNA biosynthesis and cell growth.

EXPERIMENT

Chemicals and instrumentation

Sample of Legundi leave was obtained from UPT Materia Medica Batu. Meawnhile chemicals used for research such as surfactant polysorbate 20 (tween 20), brain heart infusion (BHI), sodium chloride, nutrient broth (NB), agar, nutrient agar (NA) McFarland standard, sulfuric acid 1%, barium chloride 1%, *Staphylococcus aureus* (ATCC- 92253), ampicillin.

Instrumentation used in this research are autoclave (Tomy ES-215), analytical balance (Shimadzu AP225WD), incubator (Lab companion JeioTech IB-11E) and gas chromatography (Shimadzu QP-2010 SE).

Essential oil isolation

A fresh-leaves sample (855 g) and water are put into a Stahl-distillation apparatus. It was heated at boiling temperatures, and the condensed essential oil was collected. The oil obtained was stored in a refrigerator for further analysis and application.

GCMS analysis

Hexane was used to dilute essential oil using 1:1 ratio. The column used in GC-MS QP2010 plus is RTX-5MS. Injector temperature of 250 ºC, column oven temperature 300 ºC, pressure 100 kPa, total flow speed 50 mL/min., and column flow speed 1.88 mL/min. The chromatogram was scanned to obtain mass spectra data. The database in the library is used as a comparison for interpreting the chemical structures and prediction. Interpretation is based on molecular weight and fragmentation patterns following their similarity index (SI) [29,30].

Antibacterial *in-vitro* evaluation

Media preparation

The solid media was prepared using a 13 g of nutrient broth (NB) and 12 g agar. It was combined and diluted with 1 L distillated water. Then, this mixture was homogenized by heating and stirring. The solution was sterilized in autoclave at ± 40 – 50 ºC. Then, it was

poured in petri cup and stored at 2-8 ºC. Meanwhile, nutrient agar (NA) media was made by diluting a 20 g NA with aquadest (1 L). The solution of sodium chloride was made by combining 0.9 g of NaCl with a 100 mL of aquadest. This solution was applied for preparation of bacterial according to McFarland standard.

Purification of test bacteria

The sterilized media was heated until ± 40-50 ºC and poured in a sterile petri dish for about ± 20 minutes. Then, inoculum of *S. aureus* was inoculated into a petri dish and incubated at 37 ºC for 24 hours.

Rejuvenation of test bacteria

A single colony that grows on the cup media was taken with sterile swab cotton, inoculated in the NA media on the petri dish in a zig-zag pattern. The treatment was carried out in an aseptic laminar and incubated at 37 °C for 24 hours.

McFarland standard solution

McFarland 0.5 standard solution used was equivalent to 1.5 x 10^8 cell/mL. It was obtained from mixing of 0.05 mL of 1% $BaCl_2$ and 9.95 mL of 1% H_2SO_4.

Kirby-Bauer disc diffusion method

The procedure to evaluate antibacterial of the essential oil of *V. trifolia* following the general procedure for diffusion technique [21,22]. The media was prepared by adding the media and the bacteria in the test tube. The growth bacteria were poured on the petri dish and placed it in an oven at 37 ºC. The paper disc was inserted into a sample, control, and reference solution in a 1.5 mL microtube. The evaluated sample prepared in concentration of 2.5, 5.0, 10.0, and 20 mg/mL, respectively. Positive control was commercial antibiotic (ampicillin) solution and negative control contain solvent. The sample were incubated for 24 hours at 37 ºC and it was observed by measuring the inhibition zone on the paper disc.

Computation *in-silico* evaluation

The major identified compound from *V. trifolia* essential oil was evaluated for *in-silico* study. The spatial data file of each structure was generated from https://pubchem.ncbi.nlm.nih.gov as .SDF format. Meanwhile, the macromolecules protein as receptors from bacterial cells of *S. aureus* was obtained from the protein data bank (PDB) accessed in https://www.rcsb.org. The file format was determined as .pdb format. Visualization of solvent and ligands was performed using PyMOL software [31]. Ampicillin from the lactam group was used as a positive control. The virtual molecular docking study was conducted using Pyrx 0.9 software [32] and Vina software version 1.2.3 [33]. The docking result were visualized using Biovia software.

RESULT AND DISCUSSION

Essential oil isolation

Isolation of essential of *V. trifolia* was undergone following hydro-distillation method. The method previously already reported for volatile compound isolation [34,35]. The fresh leaves of Legundi (855 g) provide 0.13% yield of a colorless oil. Similar result was also reported previously in between 0.15 and 0.21% [36]. Variation of the essential oil quantity was correlated to environment conditions such as drought and essential oil metabolism [37]. Water content in soil increases the essential oil metabolism by increasing

phosphoenolpyruvate (PEP) carboxylase, geraniol dehydrogenase enzyme, geraniol and citral biogenesis [38].

GCMS analysis

Analysis of essential oil from *V. trifolia* underwent with GCMS. Tabulation of detected compound was summarized in Table 1. It was derived from the percentage area of each peak in the chromatogram, and the detected compound was matched with library database following mass spectra pattern and similarity index (SI) [29].

Table 1. Major compounds of essential oil composed in *V. trifolia*

No	Compound	Retention time (min.)	Percentage (%)	Similarity Index (%)
1	Sabinene	12.231	13.17	97
2	Eucalyptol	14.249	23.07	94
3	Terpinen-4-ol	19.714	4.26	89
4	α-Terpineol	20.225	5.19	95
5	Limonene	25.691	7.25	88
6	Caryophyllene	27.924	14.72	96

The identified compound i.e.: sabinene (13.17%), eucalyptol (23.07%), terpinen-4-ol (4.26%), α-terpineol (5.19%), d-Limonene (7.25%), and caryophyllene (14.72%). These compounds fragmentation patterns are similar to the previously reported (Figure 1) as sabinene [1-3], eucalyptol [4,5], terpinen-4-ol, α-terpineol, limonene [36], and caryophyllene [1,4].

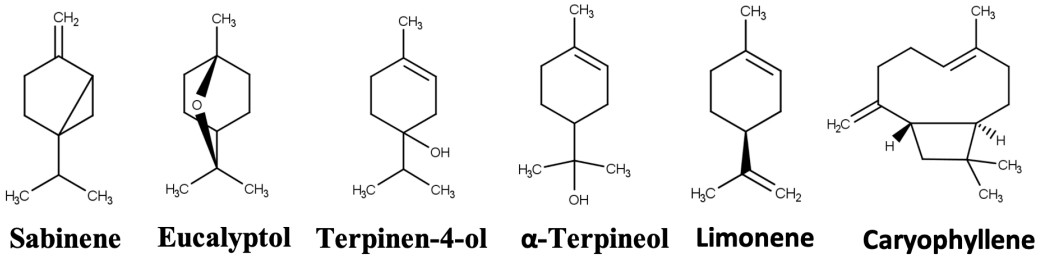

Figure 1. Molecular structure of essential oil compound in *V. trifolia*

Antibacterial evaluation *in-vitro*

Evaluation of the essential oil isolated from *V. trifolia* to inhibit the growth of *S. aureus* was summarized in Table 2. The oil concentrations were varied in between 2.5 mg/mL and 20 mg/mL. The data indicate improvement of the activity by increasing of concentration. At 2.5 mg/mL of the oil concentration, inhibit the bacterial growth by 2.94 ± 0.19 mm. Meanwhile, at 20.0 mg/mL concentration inhibit the growth by 6.91 ± 0.03 mm. This antibacterial activity shows a higher report than that using ampicillin, 0.54 ± 0.76 mm. Even though using the lowest concentration of *V. trifolia* essential oil.

This finding was in agreement [12,13,15] to the previous reported using essential oil from different species plants [14,43]. The representative photograph for disc diffusion evaluation of *V. trifolia* essential oil on *S. aureus* was displayed in Figure 2. The EO is essential oil of *V trifolia* in different concentrations, such as 2.5 mg/mL, 5.0 mg/mL, 10

mg/mL, and 20 mg/mL respectively. The essential oil from *V. trifolia* (5-20 mg/mL) exhibits intermediate inhibition against *S. aureus* because the inhibition zone is more than 5 mm. However, *S. aureus* is not susceptible when treated with 2.5 mg/mL EO. The MIC of eucalyptol is better than α-terpineol with a value of 2% and 1% against *S. aureus*, respectively. Sabinene has low antibacterial activity against *S. aureus* with an IC_{50} of 91.5 ± 13.6 [44]. Limonene exerts high inhibition activity on *S. aureus* because it can damage the permeability and integrity of cell membrane and cell wall, interfere with ATP production and decomposition, and inhibit bacterial respiration [45]. In addition, caryophyllene exerts high antibacterial activity on *S. aureus* with MIC of 3 µM compared to kanamycin as positive control (MIC 8.0 ± 2.3) [46]. Because of the low concentration of limonene and caryophyllene in the *V. trifolia* essential oil makes them unable to increase the inhibition zone of EO against *S. aureus,* significantly.

Table 2. Diameter inhibiting zone (mean ± SD) of *V. trifolia* essential oil (EO) in *S. aureus.*

Sample	Inhibition zones of sample (mm)
Negative control	0.00
Ampicillin	0.54 ± 0.76
EO 20.0 mg/mL	6.91 ± 0.03
EO 10.0 mg/mL	5.41 ± 0.25
EO 5.0 mg/mL	5.01 ± 0.51
EO 2.5 mg/mL	2.94 ± 0.19

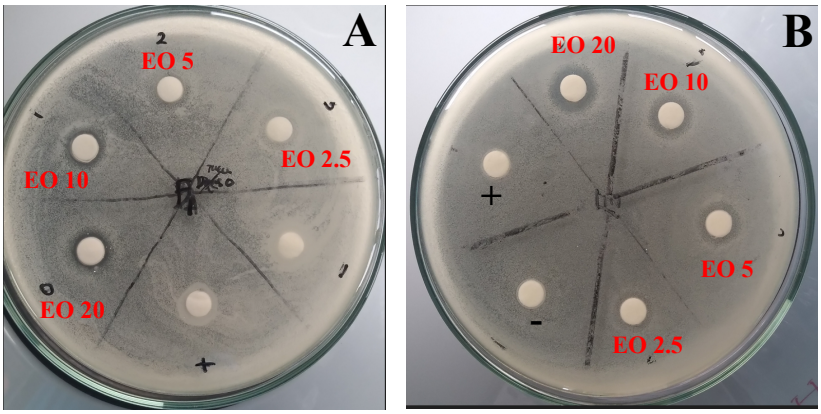

Figure 2. Inhibition zones of EO against *S. aureus*. A and B represents two repetitions.

Computational *in-silico* evaluation

Evaluation for *in-silico* study toward the major compound identified composed in *V. trifolia* is displayed in Table 3. The binding interaction was studied on tyrosyl-tRNA synthetase from *S. aureus* (PDB ID: IJIJ) (Figure 3), sortase A family protein (PDB ID: 2MLM) isolated from *S. aureus* (Figure 4), and the dihydrofolate reductase protein (PDB ID: 3FRA) isolated from *S. aureus* F98Y (Figure 5). The tyrosyl-tRNA synthetase function to catalyze tyrosine activation and its transfer into tRNA [24]. Disrupting of this biosynthesis decline in amino acid synthesis and inhibit the bacterial growth. In addition, sortase A protein

correlate to the cell wall in the pilus formation at initial level [26]. The interaction can occur at the surface of cell wall as an anchor. Disrupting this protein affect the wall formation or uptake the nutrient from outer cell. Meanwhile for dihydrofolate reductase is a protein to catalyze in the reduction of dihydrofolate [27,28] into tetrahydrofolate. This compound correlate to DNA synthesis and DNA methylation. Inhibiting this step led to cell death mechanism.

Table 3. Molecular docking of compounds from the essential oil of *V. trifolia* and native ligand with *S. aureus* proteins.

Compounds	Protein's PDB ID	Binding affinity (kcal/mol)	Bond types	Residue interactions
Native ligand	1JIJ	-8.4	Hydrogen bonds	His50, Asp195, Gly38, Gln196, Asp177, Tyr36, Tyr170, Asp80, Asp40
			Hydrophobic bonds	ALa39, Gly193, Leu70, Gln174, Thr75, Gly49
	2MLM	-6.4	Hydrogen bonds	-
			Hydrophobic bonds	Val110, Ile141, Ile124, Cys126, Arg139, Trp136, Val 108
	3FRA	-9.2	Hydrogen bonds	-
			Hydrophobic bonds	Leu28, Ile50, Ser49, Gln19, Thr46, Gln95, Thr121, Gly94, Ala7, Ile4, Tyr98, Val6, Leu20, GLy15
Ampicillin	1JIJ	-9.1	Hydrogen bonds	Tyr36, Gly38, Gln196
			Hydrophobic bonds	Cys37, His50, Phe54, Phe53, Ala39, Gln174, Leu70, Asp40, Thr75, Tyr170
	2MLM	-5.8	Hydrogen bonds	Glu113
			Hydrophobic bonds	Ala60, Arg139, Val108, Thr122, Ile141, Ile124, Ala46
	3FRA	-8.4	Hydrogen bonds	Ala7
			Hydrophobic bonds	Leu5, Val6, Val31, Phe92, Leu28, Asp27, Leu20, Thr46, Asn18, Gln19, Gly15, Thr121, Tyr98
Sabinene	1JIJ	-4.8	Hydrogen bonds	-
			Hydrophobic bonds	Asn124, Gln174, Asp177, Ala39, Tyr36, Gln196, Gly38, Thr75
	2MLM	-5.2	Hydrogen bonds	-
			Hydrophobic bonds	Val110, ile141, Arg139, Val 108, Ile124, Leu111
	3FRA	-5.6	Hydrogen bonds	-
			Hydrophobic bonds	Ala7, Val131, Leu28, Val16, Leu20, Asp27, Phe92
Eucalyptol	1JIJ	-4.9	Hydrogen bonds	Asp40
			Hydrophobic bonds	Ala39, Thr42, Gln196, Asp80, Gly38
	2MLM	-4.8	Hydrogen bonds	-
			Hydrophobic bonds	Leu111, Val110, Ile141, Ie124, Arg139, Val108
	3FRA	-5.8	Hydrogen bonds	-
			Hydrophobic bonds	Ile14, Gln19, Leu20, Asn18, Gly94, Thr46, Phe92, GLy93,
Terpinen-4-ol	1JIJ	-5.9	Hydrogen bonds	Gln174, Gln196
			Hydrophobic bonds	Tyr36, Gly38, Asp40, Ala39
	2MLM	-5.0	Hydrogen bonds	Val108
			Hydrophobic bonds	Val110, Ile141, Arg139, Ile124, Glu113,

				Leu111
	3FRA	-6.1	Hydrogen bonds	Leu5, Phe92, Tyr98
			Hydrophobic bonds	Leu20, GLy94, Ala7, Ile14, Val31, Val6, Asp27
α-Terpineol	1JIJ	-6.5	Hydrogen bonds	Asp40, Tyr170, Thr75
			Hydrophobic bonds	Tyr36, Gln196, Val91, Gln190, Gln174, Gly38, Ala39, Asn124, Asp177, Leu70
	2MLM	-5.0	Hydrogen bonds	Leu111, Glu113, Val108
			Hydrophobic bonds	Ile141, Arg139, Ile124
	3FRA	-6.0	Hydrogen bonds	Leu5, Tyr98, Phe92
			Hydrophobic bonds	Ile50, leu28, Val131, Ala7, Val6
Limonene	1JIJ	-5.7	Hydrogen bonds	-
			Hydrophobic bonds	Thr75, Ala39, Gly72, Leu70, Asp40, Gln196, Val91, Tye36, Gln190, Ile200, Gln174, Gly38, Asp177
	2MLM	-4.9	Hydrogen bonds	-
			Hydrophobic bonds	Glu113, Ile124, Ile141, Arg139, Val110, Val108, Leu111
	3FRA	-5.5	Hydrogen bonds	-
			Hydrophobic bonds	Asp27, Leu5, Phe95, Leu20, Ile14, Tyr98, Ala7
Caryophyllene	1JIJ	-5.8	Hydrogen bonds	-
			Hydrophobic bonds	Tyr170, Gly38, Gln196, Ala39, His50, Asp195, Asp40, Lys84, Asp80
	2MLM	-5.8	Hydrogen bonds	-
			Hydrophobic bonds	Val108, Arg139, Ile124, Ile141
	3FRA	-7.5	Hydrogen bonds	-
			Hydrophobic bonds	Leu5, Val131, Leu28, Ala7, Leu20, Ile50, Thr46, Phe92, Asp27

Note: Native ligand applied for 1JIJ: 629 ([2-amino-3-(4-hydroxy-phenyl)-propionylamino]1,3,4,5-tetrahydroxy-4-hydroxy-methyl-piperidine-2-yl)-acetic acid); 2MLM: 2w7 (N-(2-oxo-2-[3S,5S,7S-tricyclo[3.3.1.1-3,7-]dec-1-ylamino]ethyl)-2-sulfanylbenzamide), and 3FRA: i2h (5-(((2S-)-2-cyclopropyl-7,8-dimethoxy-2H-chromen-5-yl)methyl) pyrimidine-2,4-diamine).

Aminoacyl-tRNA synthetase enzyme in *S. aureus* has an important role in catalyzing the formation of adenosine triphosphate (ATP) that contributes to nucleic acid synthesis. It is important role, potent antibacterial drug specifically designed for inhibiting the active site of this enzyme. The most important synthetase class belongs to class I synthetase, tyrosyl-tRNA synthetase (TyrRs) [47]. All essential oil compounds do not have a comparable binding affinity with tyrosyl-tRNA synthetase (Table 3). The best compound to inhibit this enzyme is α-terpineol (-6.5 kcal/mol) because it inhibits the same active site as the native ligand and ampicillin (Figure 3). The main residues that interact with both α-terpineol and native ligand are Tyr170, Thr75, Tyr36, Gln196, Gln174, Gly38, Ala39, Asp177, Leu70.

Sortase A plays a role in connecting virulence factors via covalent bonds to the bacterial cell wall. The virulence factors in *S. aureus* contain protein A, microbial surface components that can identify adhesive matrix compounds and other proteins that bonded covalently to the peptidoglycan of cell walls [48]. Inhibiting sortase A leads to disrupting protein adhesion to the cell wall [49]. Thus, it is important target for antibacterial agents. Caryophyllene (-5.8 kcal/mol) has a similar binding affinity to ampicillin (-5.8 kcal/mol), so it can be used to replace ampicillin to inhibit sortase A of *S. aureus*. Both inhibit the same

active site as the native ligand (Figure 4) with similar interactions for residue Val108, Arg139, Ile124, and Ile141.

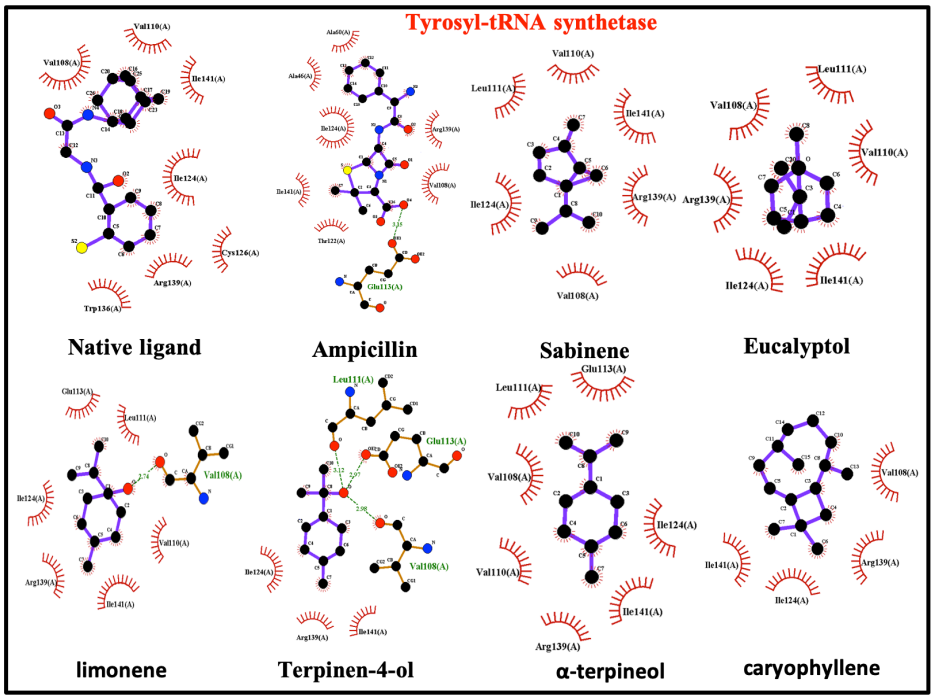

Figure 3. Interaction between compound from *V. trifolia* essential oil and tyrosyl-tRNA synthetase.

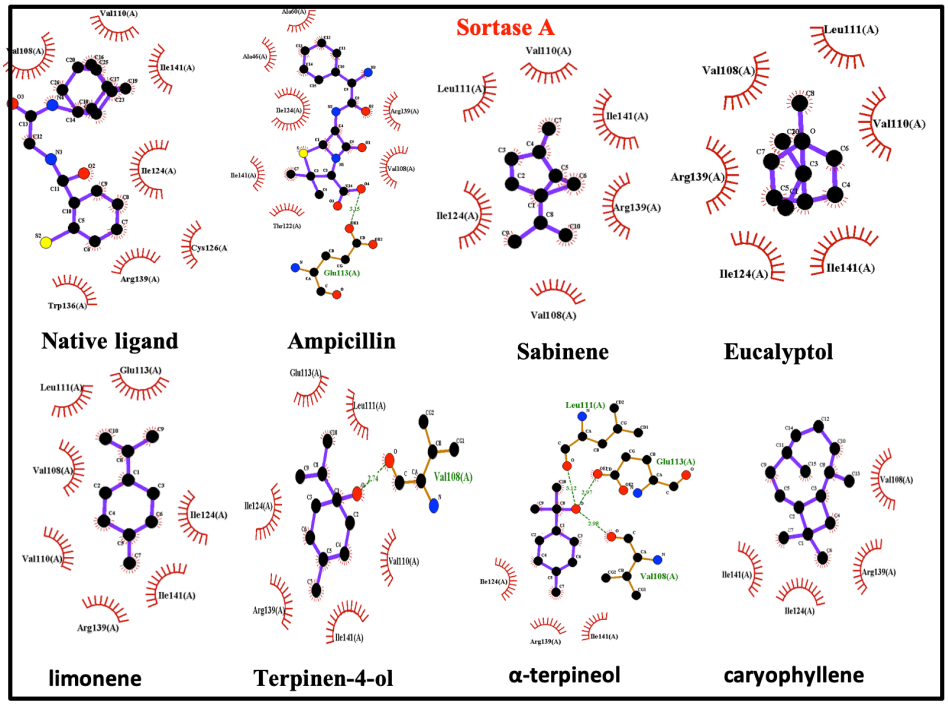

Figure 4. Interaction between *V. trifolia* essential oil and sortase A.

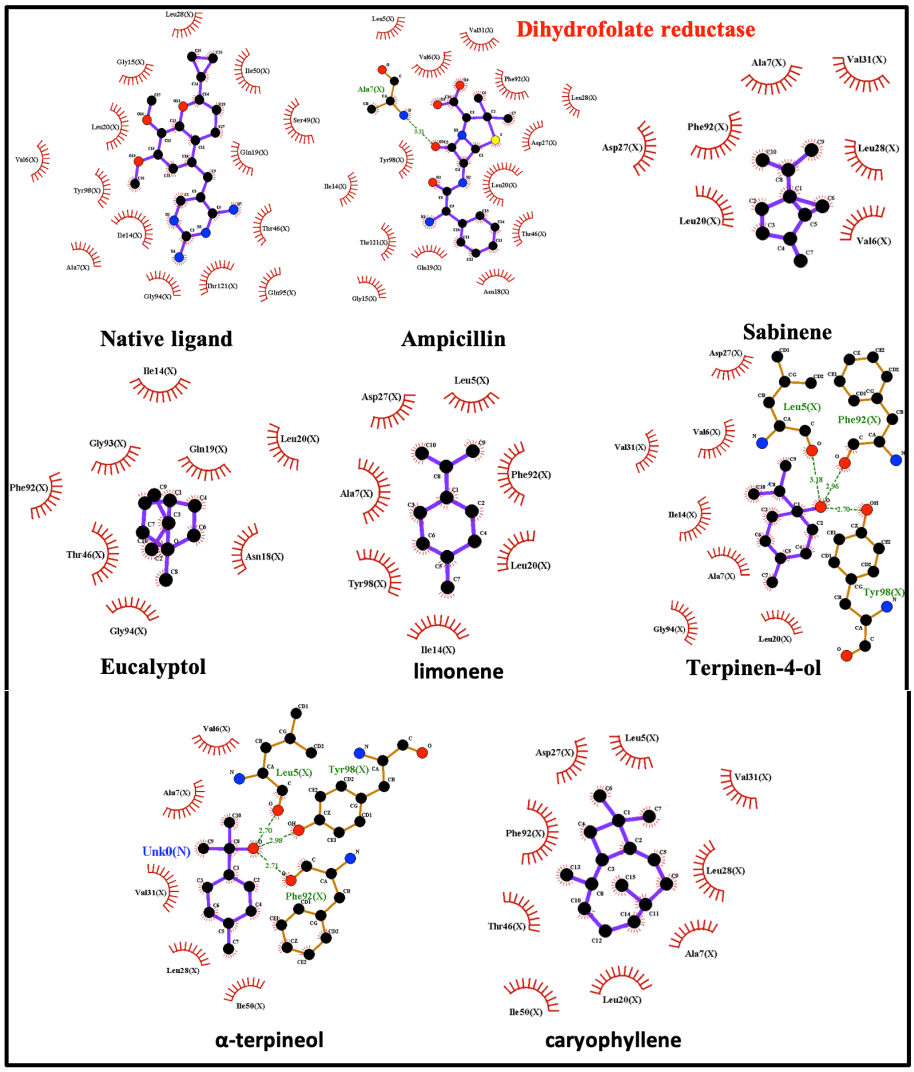

Figure 5. Interaction between compound from the essential oil of *V. trifolia* and dihydrofolate reductase.

Dihydrofolate reductase (DHFR) is the main enzyme in 5,6,7,8-tetrahydrofolate (THF) synthesis pathway. THF is an essential cofactor in several biosynthesis pathways of methionine, thymidylate, and pantothenate. Disrupting this enzyme by its active site inhibition will lead to cell death. Essential residues in the DHFR active site are Leu20, Val31, Ile50, and Leu54 [50]. Only ampicillin (-8.4 kcal/mol) has close binding energy to the native ligand (-9.4 kcal/mol), followed by caryophyllene (-7.5 kcal/mol), terpinen-4-ol (-6.1 kcal/mol), α-Terpineol (-6.0 kcal/mol), eucalyptol (-5.8 kcal/mol), sabinene (-5.6 kcal/mol), and limonene (-5.5 kcal/mol). Both ampicillin and caryophyllene are located within the same active site as the native ligand (Figure 5). Caryophyllene interacts with crucial residues of DHFR, such as Val131, Leu20, and Ile50.

The molecular docking study indicate that α-terpineol and caryophyllene were the best compound composed in the essential oil from *V. trifolia* leaves to have binding activity values. Both compounds potent to inhibit tyrosyl-tRNA synthetase, sortase A and dihydrofolate reductase. The strong interaction toward the protein hampers and disrupts the

protein activity. Thus, play main role in the antibacterial activity toward *S. aureus* from *V. trifolia* essential oil.

CONCLUSION

The essential oil isolated from *V. trifolia* leaf shows inhibition towards *S. aureus* with caryophyllene (-7.5 kcal/mol against dihydrofolate reductase) and α-terpineol (-6.5 kcal/mol against tyrosyl-tRNA synthetase) as the main compounds that responsible for its antibacterial activity. Other compounds such as sabinene, eucalyptol, terpinen-4-ol, and limonene give a lesser binding affect toward antibacterial activity. This finding opens the way for future understanding in application study.

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