In Vitro and In Silico Characterization of Antimalarial Myristicyl Propanoate

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Abstract. Myristicyl propanoate is an ester derived from myristicin, the most aromatic compound of Nutmeg (Myristica fragrans) which has been successfully synthesized. The myristicyl propanoate compound in this study was characterized in terms of its antimalarial activity in vitro and in silico using P. falciparum and molecular docking studies of the Dihydrofolate Reductases-Thymidylate Synthase (DHFR-TS) enzyme, respectively. IC₅₀ value of myristicyl propanoate as 751.96 µg mL⁻¹, while the binding energy value of ∆G myristicyl propanoate ligand with receptors was -5.44 kcal mole⁻¹ and the value of inhibition constant (Ki) was 103509 nM. Hydrogen bonding between the test ligand with the DHFTR-TS enzyme occurred in one amino acid residue TRP48, and the π bond was observed in the amino acid residue PHE⁵⁸. In addition, hydrophobic bonds occurred in several amino acid residues such as THR¹⁸⁵, TRY⁵⁷, CYS¹⁵, ILE¹⁶⁴, ILE¹⁴, TYR¹⁷⁰, ASP⁵⁴, MET⁵⁵, ALA¹⁶, LEU⁴⁶. The results of characterization in vitro and in silico of the myristicyl propanoate compounds showed that the compounds are inactive as an antimalarial.

1. Introduction
Malaria is one of the endemic diseases in tropical and subtropical countries [1]. Malaria is an infectious disease caused by Plasmodium. Malaria is transmitted through the bite of a female Anopheles mosquito that contains Plasmodium [2]. Infection caused by malaria parasites is still a problem in many countries such as Africa and Asia, including Indonesia [3]. The World Malaria Report 2015 states that malaria has affected 106 countries in the world. A global commitment to Sustainable Development Goals (SDGs), which seeks to eradicate malaria by ending the endemic of the disease until 2030 [2].

The challenge in treating malaria is drug resistance. Chloroquine resistance to Plasmodium falciparum, first occurred in 1950 in the Thailand-Cambodia area, until now it has been reported resistance to all types of malaria in Africa. Mefloquine was reported resistance in Asia. Sulfadoxine-pyrimethamine as a substitute for chloroquine in Africa is now beginning to decrease its effectiveness progressively. New groups of anti-microbial drugs that have the potential to inhibit P. falciparum are artemisinin (1a) and their derivatives (1b-c) [4]. Artemisinin (1a) is a lactone sesquiterpene type chemical compound, a cyclic ester [5].
In vitro evaluation of antimalarial activity shows that Nutmeg (Myristica fragrans) can inhibit *P. falciparum* with IC$_{50}$ below 10 μg mL$^{-1}$ [6]. The main aromatic compound of nutmeg is myristicin (2) [7]. Myristicin can be synthesized into its derivative compound, which is myristicinyl propanoate (3) [8]. The presence of an ester group is thought to increase its potential as an antimalarial.

In addition to *in vitro* studies, one of the ways that can be used in finding new malaria candidates can be done by in silico (molecular docking) studies. The method is carried out with the help of a computer (Computer Aided-Drug Discovery), making it more cost-effective [9]. In this study, we focus on *in vitro* antimalarial studies of the myristicinyl propanoate (3) compound against *P. falciparum*. While *in silico* antimalarial studies of natural inhibitors used are the enzyme dihydrofolate reductase-thymidylate synthase (DHFR-TS). These enzyme play a role in the multiplication of *Plasmodium* cells [10-11].

2. Methodology

2.1 Tools and Materials

Hardware tools in the form of one MSI GL-63 laptop computer (Intel Core I5-8300H, 12GB DDR4 RAM, 256GB SSDNVME, and Nvidia Geforce GTX 1050 VGA) and Windows 10 Home operating system software, Marvin Sketch® 16.9.12, AutoDock Tools®4.2, AutoDock Vina®1.1.2, VMD 1.9.2, Ligplot + 4.5.3, PyMOL 2.3 and Discovery Studio 19.0 Client (DSV 19.0).

The materials used in this study are myristicinyl propanoate (3) compounds from previous studies, media (Roswell Park (RP), NaHCO$_3$ 5%, transport media and sorbitol for synchronization, blood serum, erythrocytes from blood group O and culture of *P. falciparum* stock.

2.2. Antimalarial Activity Test *in vitro* against *P. falciparum*

Antimalarial activity tests were carried out *in vitro*. Before testing the effectiveness of the compound myristicinyl propanoate (3), the parasite breeding was carried out continuously with the candle jar method developed by Trager and Jensen. The activity began with the manufacture of media (Roswell Park (RP), NaHCO$_3$ 5%, transport media and sorbitol for synchronization, blood serum, erythrocytes from blood group O and stock culture of *P. falciparum*) [12].

After 24 hours of incubation, *P. falciparum* cultures were removed and blood smear preparations were made. Making blood preparations was done by dripping 1 drop of myristicinyl propanoate (3) with various concentrations (0.01; 0.05; 0.1; 0.5; 1; 5,10,50, 100, 200 μg mL$^{-1}$) on top glass object, then made thin and thick smear. Samples were left to dry overnight. Thin blood preparations were fixed
with methanol for 1 second, then stained with Giemsa solution by standard (3% for 45 minutes) and rinsed using running water. Blood preparations were examined under a microscope at magnification 1000 times using immersion oil. The reading began with a thick blood preparation to see the development of the parasitic growth as a whole prediction of compound activity on the DHFR-TS enzyme.

2.3. Identification of Malaria Parasite Receptors
The DHFR-TS enzyme, the target protein structure data and its binding site from the results of previous studies, was obtained through Protein Data Bank (PDB) with GDP ID: 1J3I along with the original ligand. The three-dimensional structure of the ligand, myristicyl propanoate (3), was drawn with Marvin sketch®16.9.12 and its structure was optimized as a test compound. The tested protein was then carried out by the identification process carried out using VMD 1.9.2 software.

2.4. 3D Structure Optimization of Malaria Parasite Receptors
The structure optimization process was conducted using the ADT 1.5.6 and PyMOL 2.3 software. The initial stage was to remove water molecules (H₂O) around proteins, heteroatoms and natural ligands using PyMOL. Next is the addition of a Gasteiger and Hydrogen charge with ADT 1.5.6, then the file was saved in PDBQT format.

2.5. 3D Ligand Structure Optimization
The ligand used for this study was myristicyl propanoate (3), which was designed and geometrically optimized by finding the lowest energy conformer using Marvin sketch software. The ligand structure was stored in PDB storage format. The next step was to add the Gasteiger and Hydrogen load using ADT 1.5.6. The obtained file then was saved in PDBQT format.

2.6. Docking Parameter Validation Process
The docking parameter validation process was carried out using ADT 1.5.6. Validation was done by docking the natural ligands attached to the protein used as docking objects. The natural ligand was attached to the receptor is 1,6-dihydro-6,6-dimethyl-1-[3-(2,4,5-trichlorophenoxy) propoxy]-1,3,5-triazine-2,4 diamine (WR99210). Natural ligand files were prepared by extracting their structure from the receptor using PyMOL. The extracted natural ligand file was then merged with nonpolar hydrogen and then stored in PDBQT format. Next, the receptors are combined with natural ligands and continued with the grid process and the docking parameters are entered. The docking process produced ten natural ligand poses. Next, the conformation pose analyzed to find the pose that is most similar to the natural ligand. The most similar pose will have the lowest RMSD value. The RMSD value must be <2 to be valid as a docking parameter for the test compound.

2.7. Molecular Docking Process
The grid process was carried out using the ADT 1.5.6, molecular tethering was done with AutoDockVina (Scripps Research Institute, USA). The grid size and center size were rearranged so that the area of the box covers the entire active site of the receptor. Exaustiveness was set at number 100. The vina folder was placed on the C: \ Vina drive and then filled in the CONFIG.TXT file with the parameters above in accordance with the center number and its size.

Tethering commands are executed through the CMD window, vina.exe is called via the "C: \ vina -- config conf.txt --log log.txt" command, then the enter key is pressed and the process is finished. The ligands in which have the smallest Gibbs (ΔG) free energy from the list are stored in PDBQT format (ADT 1.5.6). Then the pose is combined with the receptor and stored in PDB format using DSV 19.0.

2.8. Analysis
The bond energy value (ΔG) and the docking RMSD value were analyzed using log file data from AutoDockVina. The binding residue and the hydrogen ligand-receptor bond were analyzed in 2D
using Ligplot + 4.5.3. Pi (π) bonds and visualize the 3D structure of the ligand-receptor were analyzed using the DSV 19.0 Software.

3. Results and Discussion

3.1. Antimalarial Activity Test in vitro against *P. falciparum*

Research on antimalarial activity of myristicyl propanoate (3) in vitro with *P. falciparum* was carried out as a preliminary test to evaluate the activity of these compounds as antimalarial. *P. falciparum* is a cause of malaria in humans which has several strains that are resistant to many antimalarial drugs [13]. The results of the antimalarial test of myristicyl propanoate compound (3) against *P. falciparum* in vitro are shown in Table 1.

| Levels (µg mL⁻¹) | Parasitemia | Percentage of average inhibition (%) | IC₅₀ (µg mL⁻¹) |
|------------------|-------------|--------------------------------------|----------------|
| Control negatif  | 5.6         | 0                                    |                |
| 200              | 4.73        | 15.48                                |                |
| 100              | 3.53        | 36.90                                |                |
| 50               | 4.37        | 22.02                                |                |
| 10               | 4.97        | 11.31                                |                |
| 5                | 4.33        | 22.62                                | 751.96         |
| 1                | 4.97        | 11.31                                |                |
| 0.5              | 1.07        | 2.38                                 |                |
| 0.1              | 5.83        | 4.17                                 |                |
| 0.05             | 6.27        | 11.90                                |                |
| 0.01             | 5.27        | 5.95                                 |                |

Table 1: Test results of Antimalarial myristicyl propanoate (3) against *P. falciparum*.

Table 1 showed that the compound myristicyl propanoate (3) was only able to kill 2-37% *P. falciparum*. The compound (3) is not acting as an antimalarial as indicated by the IC₅₀ value of 751.96 µg mL⁻¹. A drug ingredient is said to be active as anti-malaria if it has an IC₅₀ value <25 µg mL⁻¹ [14]. The *in vitro* test results are very different from the antimalarial activity of Nutmeg (*M. fragrans*), which actively inhibits *P. falciparum* with IC₅₀<10 µg mL⁻¹ [6]. Although myristicyl propanoate is a derivative of myristicin (2), the main aromatic compound of nutmeg [7]. This could be due to compounds that are active against malaria in nutmeg instead of myristicin compounds.

3.2. Docking Parameter Validation

One method of validating docking parameters is to docking back natural ligands on the active site of the target protein that has been eliminated by natural ligands (self-docking) [15]. Parameters are said to be valid if the docking parameters designed can tether natural ligands or congenital ligand complexes to their original position. Validation of docking parameters was done by docking WR99210 to the pfDHFR-TS enzyme with parameters namely center_x = 27.67, center_y = 6.648, center_z = 58.203, grid box size namely size_x = 40, size_y = 40, size_z = 40 and spacing = 0.266.

Validation results showed that an RMSD value of 0.78, indicating that the parameters used are good enough to tether WR99210 to its original position. The smaller RMSD value shows the similarity of the poses of the two ligands when overlapping is getting bigger. In addition, WR99210 interacted with the same amino acids before the ligand was re-tethered, namely TYR¹⁷⁰, ILE¹⁴, ILE¹⁶⁴, CYS¹⁵, PHE⁸*, ASP⁶⁴, MET⁵⁵, PRO¹¹³, ILE¹¹².
3.3. Prediction of Myristicyl Propanoate (3) Compound Activity Against DHFR-TS enzyme in silico with Molecular docking

Docking studies were used extensively in drug discovery as in the prediction of the structure of the receptor-ligand complex and also to rank the ligand molecule based on the binding energy of the enzyme-ligand complex. Our docking study aimed to find out the interaction between myristicylpropanoate (test ligand) with DHFR-TS enzyme compared with WR99210 (natural ligand) interaction. WR99210 is a comparative ligand because it can actively inhibit the pfDHFR-TS enzyme. The results obtained from the doctor's results are detailed in Table 2.

| Compound                   | Thermodynamic parameters | Amino Acid Residues that interact and bond types |
|----------------------------|--------------------------|--------------------------------------------------|
|                            | Bonding energy (kcal mole$^{-1}$) | inhibition constant Ki (nM) | Hydrogen bonding | II bonding | Hydrophobic interactions |
| WR99210 (Natural Ligand)   | -8.83                    | 335.02                           | ILE$^{164}$, ILE$^{14}$, TYR$^{170}$, ASP$^{54}$, ALA$^{16}$, CYS$^{15}$, PHE$^{58}$, LEU$^{119}$, PRO$^{113}$, PHE$^{116}$, ILE$^{112}$ |
| Myristicyl propanoate (3) (Test Ligand) | -5.44                   | 103509                           | TRP$^{48}$, PHE$^{58}$, THR$^{185}$, TRY$^{57}$, CYS$^{15}$, ILE$^{164}$, ILE$^{14}$, TYR$^{170}$, ASP$^{54}$, MET$^{55}$, ALA$^{16}$, LEU$^{46}$ |

Table 2: Interactions between ligands and DHFR-TS enzymes.

Table 2 showed the binding energy value of $\Delta G$ myristicyl propanoate (3) ligand with a receptor of -5.44 kcal mole$^{-1}$, while the binding energy value of $\Delta G$ WR99210 natural ligand as a comparison with a receptor of -8.83 kcal mole$^{-1}$. The binding energy value of $\Delta G$ myristicyl propanoate (3) ligand was smaller when compared with $\Delta G$ WR99210. The binding energy describes the magnitude of the stability of the bond between the enzyme and the substrate. The smaller the value of the binding energy is the more stable the bond. This result showed that the bond between the natural ligand and the receptor is more stable compared to the test ligand when it binds to the active site of the enzyme. This result was also supported by the value of the inhibition constants (Ki) of the myristicyl propanoate (3) test ligand (103509 nM) which was greater than the natural ligand (335.02 nM). The smaller the value of Ki, the stronger the bond between the inhibitor and the enzyme.

The difference in binding energy value and Ki value between natural ligands and myristicyl propanoate (3) test ligands was due to differences in amino acid residues that interact with ligands, resulting in differences in interactions between molecules. These interactions include $\Pi$ ($\pi$) bonds, hydrogen bonds, and hydrophobic interactions. The interaction WR99210 natural ligand and myristicyl propanoate (3) test ligand with the substrate is shown in Figure 1 and 2.

Figure 1a showed that the hydrogen bond between the natural ligand and the DHFTR-TS enzyme occurred in amino acid residue ILE$^{164}$, ILE$^{14}$, TYR$^{170}$, and ASP$^{54}$. The $\pi$ bond was observed in the amino acid residues of ALA$^{16}$, CYS$^{15}$, PHE$^{58}$, LEU$^{119}$, PRO$^{113}$, PHE$^{116}$, while hydrophobic bonds only occurred in one amino acid residues, namely as the ILE$^{112}$. The hydrogen bonding between natural ligands and several amino acid residues was greater than the hydrophobic interactions, resulting in smaller binding energy. Figure 1b showed that the hydrogen bond between the test ligand and the DHFTR-TS enzyme was notified in amino acid residue, TRP$^{48}$ and in the atomic O in the carbonyl group of the test compound. The $\pi$ bond occurred in the amino acid residue PHE$^{58}$ with an aromatic
ring. Hydrophobic bonds were found in several amino acid residues such as THR$_{185}$, TRY$_{57}$, CYS$_{15}$, ILE$_{64}$, ILE$_{14}$, TYR$_{170}$, ASP$_{54}$, MET$_{55}$, ALA$_{16}$, LEU$_{46}$. The number of hydrophobic interactions that occur is thought to result in the large energy value of $\Delta G$ myristicyl propanoate (3) ligand with receptors. This was the opposite of the comparison ligands, which have more hydrogen bonding interactions with amino acid residues compared to their hydrophobic interactions (Table 2). The in silico test results supported the in vitro test results of *P. falciparum*, the myristicyl propanoate (3) test ligand as inactive as an antimalarial.

![Interaction of 2D natural ligand (a) and test ligand (Myristicyl propanoate) (b) with amino acid residues on the active site of the DHFR-TS enzyme](image)

Figure 1: Interaction of 2D natural ligand (a) and test ligand (Myristicyl propanoate) (b) with amino acid residues on the active site of the DHFR-TS enzyme
Figure 2: Interaction of 3D test ligands (Myristicyl propanoate) with amino acid residues on active site of DHFR-TS enzymes

4. Conclusion
The characterization of the myristicyl propanoate compound in vitro against P. falciparum and in silico using molecular docking showed that the compound is inactive as an antimalarial. IC$_{50}$ value of myristicyl propanoate as 751.96 $\mu$g mL$^{-1}$, while the binding energy value of $\Delta$G myristicyl propanoate ligand with receptors was -5.44 kcal mole$^{-1}$ and the value of inhibition constant (Ki) was 103509 nM. Hydrogen bonding between the test ligand with the DHFTR-TS enzyme occured in one amino acid residue TRP48, and the $\pi$ bond was observed in the amino acid residue PHE$_{58}$. In addition, hydrophobic bonds occured in several amino acid residues such as THR$_{185}$, TRY$_{57}$, CYS$_{15}$, ILE$_{164}$, ILE$_{41}$, TYR$_{170}$, ASP$_{54}$, MET$_{55}$, ALA$_{16}$, LEU$_{46}$. The results of characterization in vitro and in silico of the myristicyl propanoate compounds showed that the compounds are inactive as an antimalarial.

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