



In silico and *in vitro* antimalarial activity evaluation of eugenol's transformation products against *Plasmodium falciparum* strain 3D7 using the Trager and Jensen method

[Evaluación de la actividad antipalúdica *in silico* e *in vitro* de los productos de transformación del eugenol frente a la cepa 3D7 de *Plasmodium falciparum* mediante el método de Trager y Jensen]

Galih S. Putra^{1*}, Melanny I. Sulistyowaty^{7,8}, Yahmin Yahmin¹, Sumari Sumari¹, Rini Retnosari¹, Siti Marfu'ah¹, Farida Anwari², Farida Suhud³, Dini Kesuma³, Burhan Ma'arif⁴, Rudy Mardianto⁵, Adulsman Sukkaew⁶, Takayasu Yamauchi⁸

¹Department of Chemistry, Faculty of Mathematics and Natural Sciences, State University of Malang, Indonesia.

²Pharmaceutical Chemistry Department, University of Anwar Medika, Sidoarjo, Indonesia.

³Department of Pharmaceutical Chemistry, Faculty of Pharmacy, University of Surabaya, Surabaya, Indonesia.

⁴Department of Pharmacy, Faculty of Medicine and Health Science, Maulana Malik Ibrahim State Islamic University, Malang, Indonesia.

⁵Institut Teknologi, Sains, dan Kesehatan RS.DR. Soepraoen Kesdam V/BRW, Malang, Indonesia.

⁶Faculty of Sciences, Technology and Agriculture, Yala Rajabhat University, Yala, Thailand.

⁷Department of Pharmaceutical Sciences, Faculty of Pharmacy Airlangga University, Surabaya, Indonesia.

⁸School of Pharmacy and Pharmaceutical Sciences, Hoshi University, Shinagawa City, Tokyo, Japan.

*E-mail: galih.satrio.fmipa@um.ac.id

Abstract

Context: Endemic malaria occurs in tropical areas with high morbidity and mortality. First-line therapy using chloroquine and artemisinin-based combination therapy has reported resistance; therefore, new drugs need to be developed. Eugenol, a clove-derived phenolic compound, was selected due to its modifiable hydroxyl group and prior evidence of antimalarial potential.

Aims: To develop an antimalarial drug candidate derived from eugenol transformation.

Methods: Eugenol was transformed via the Mannich reaction using formaldehyde and aniline to form iminium salts, followed by reaction with eugenol. Product screening via GC-MS was conducted after refluxing for 4, 6, and 10 hours. Purification used column chromatography (silica gel 60; n-hexane:ethyl acetate = 10:1). Compound structures were confirmed by FTIR, ¹H-NMR, and UV/Vis. ADMET screening was performed using pkCSM. Molecular docking and dynamics were conducted against plasmepsin II (PDB: 1LEE). *In vitro* antimalarial activity was tested using the Trager and Jensen method.

Results: The study showed that compounds derived from eugenol transformation had potential as an antimalarial agent, surpassing the efficacy of the native ligand and chloroquine. Additionally, achieving good results in terms of ADMET screening. IC₅₀ of the products was <10 µM, which is categorized as good activity, especially compounds **5** (6,6'-((phenylazanediy)bis(methylene))bis(4-allyl-2-methoxyphenol) and compound **2** (1,3,5-triphenyl-1,3,5-triazinane). Both compounds showed IC₅₀ <1 µM, which is categorized as excellent activity.

Conclusions: The transformation products of eugenol can be further developed as potential antimalarial agents. Compound **5** exhibited unprecedented IC₅₀ values (<0.02 µM), though its pharmacokinetic limitations warrant further optimization. Further research is focused on cytotoxic assays.

Keywords: antimalarial; eugenol; *in silico*; *Plasmodium falciparum*.

Resumen

Contexto: La malaria endémica se presenta en zonas tropicales con alta morbilidad y mortalidad. Se ha reportado resistencia al tratamiento de primera línea con cloroquina y terapia combinada a base de artemisinina; por lo tanto, es necesario desarrollar nuevos fármacos. Se seleccionó el eugenol, un compuesto fenólico derivado del clavo, debido a su grupo hidroxilo modificable y a la evidencia previa de potencial antimalárico.

Objetivos: Desarrollar un fármaco antimalárico candidato derivado de la transformación del eugenol.

Métodos: El eugenol se transformó mediante la reacción de Mannich con formaldehído y anilina para formar sales de iminio, seguida de una reacción con eugenol. El producto se cribó mediante GC-MS tras reflujo durante 4, 6 y 10 horas. La purificación se realizó mediante cromatografía en columna (gel de sílice 60; n-hexano:acetato de etilo = 10:1). Las estructuras de los compuestos se confirmaron mediante FTIR, RMN-¹H y UV/Vis. El cribado ADMET se realizó mediante pkCSM. Se realizó acoplamiento molecular y dinámica contra plasmepsina II (PDB: 1LEE). La actividad antipalúdica *in vitro* se evaluó mediante el método de Trager y Jensen.

Resultados: El estudio demostró que los compuestos derivados de la transformación del eugenol tenían potencial como agente antipalúdico, superando la eficacia del ligando nativo y la cloroquina. Además, se obtuvieron buenos resultados en el cribado de ADMET. La CI₅₀ de los productos fue <10 µM, lo que se considera una buena actividad, especialmente los compuestos **5** (6,6'-((fenilazanedii)bis(metileno))bis(4-alil-2-metoxifenol) y el compuesto **2** (1,3,5-trifenil-1,3,5-triazinano). Ambos compuestos mostraron una CI₅₀ <1 µM, lo que se considera una actividad excelente.

Conclusiones: Los productos de transformación del eugenol pueden seguir desarrollándose como posibles agentes antipalúdicos. El compuesto **5** presentó valores de CI₅₀ <0,02 µM, aunque sus limitaciones farmacocinéticas justifican una mayor optimización. La investigación se centra en ensayos citotóxicos.

Palabras Clave: antipalúdico; eugenol; *in silico*; *Plasmodium falciparum*.

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AUTHOR INFO

ORCID:

[0000-0003-4192-3621](https://orcid.org/0000-0003-4192-3621) (GSP)

[0000-0002-9510-6822](https://orcid.org/0000-0002-9510-6822) (MIS)

[0000-0002-1788-2113](https://orcid.org/0000-0002-1788-2113) (SS)

INTRODUCTION

Malaria is a highly endemic disease in Eastern Indonesia. The first-line therapies, including artemisinin-based combination therapy (ACTs) and chloroquine, have encountered resistance, necessitating the development of new antimalarial drugs (Batista et al., 2009; Khairani et al., 2021; Sulistyowaty et al., 2025; Widya-waruyanti et al., 2007). One of the promising natural compounds for antimalarial drug development is eugenol, an essential oil predominantly obtained from *Syzygium aromaticum* Linn (Gondokesumo et al., 2021; Sharma et al., 2018).

Clove oil (eugenol) contains functional groups that can be chemically modified, making it a suitable starting material for synthesizing novel and potentially more effective compounds (Gondokesumo et al., 2021; Putra et al., 2016). The hydroxyl group in eugenol can be transformed into various derivatives such as benzoxazines, aminomethyl compounds, and epoxides (da Silva et al., 2018; Gondokesumo et al., 2021; Putra et al., 2016; Rudyanto et al., 2014). Methanol extracts of clove flowers exhibit antimalarial activity *in vitro*, with an IC₅₀ value of 25 µg/mL. Transforming eugenol into a 1,3-benzoxazine derivative significantly enhances its antimalarial potency *in vitro*, yielding an IC₅₀ value of 0.49 µM (Sharma et al., 2018). In addition to its antimalarial activity, benzoxazine compounds have also been reported to exhibit anticancer, antibacterial, and antifungal activities (Kesuma et al., 2019; 2022; Sulistyowaty et al., 2021; 2023).

Various eugenol transformation products can be synthesized by reacting eugenol with aniline and formaldehyde through a Mannich reaction (Putra et al., 2016; Rudyanto et al., 2014). These studies synthesized benzoxazine derivatives of eugenol using conventional reflux heating, which required extended reaction times. In subsequent research, microwave irradiation was employed for the Mannich reaction of eugenol, achieving high yields in a significantly shorter time (Gondokesumo et al., 2021).

In this research, eugenol was reacted with Mannich bases derived from aniline and formaldehyde, and the products were monitored using GC-MS instrumentation. The eugenol transformation products were purified through column chromatography using a mobile phase of n-hexane:ethyl acetate (10:1). Structural confirmation was performed using NMR spectroscopy, followed by antimalarial testing both *in silico* and *in vitro*.

The *in silico* antimalarial tests involved molecular docking and molecular dynamics studies of the eugenol transformation products against plasmepsin II (PDB ID: 1LEE) (Asojo et al., 2002). Plasmepsin II is an enzyme in *Plasmodium sp.* that plays a key role in

hemoglobin degradation, providing amino acids essential for trophozoite development within red blood cells (De Oliveira et al., 2013; Faloye et al., 2023; Mishra et al., 2019; Sivaramakrishnan et al., 2020). Inhibiting the aspartic protease activity of plasmepsin II would impede the growth of *Plasmodium sp.* parasites (Fig. 1).

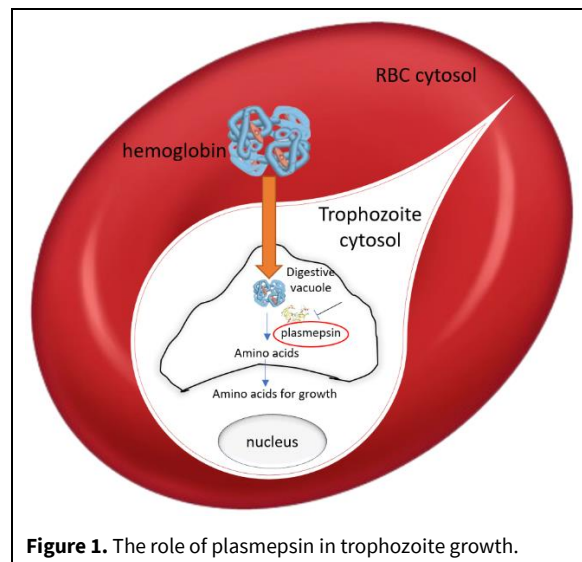


Figure 1. The role of plasmepsin in trophozoite growth.

In vitro antimalarial testing employs several methods to assess antimalarial activity by observing parasite growth within red blood cells. These methods include thick film examination, isotopic assays, quantification of parasite proteins, DNA staining and intercalation assays, the semi-automated microdilution technique, and the Trager and Jensen method (Nagueira and do Rosário, 2010).

In this research, we evaluated antimalarial activity using the Trager and Jensen method on *Plasmodium falciparum* strain 3D7, with chloroquine as the reference compound. The Trager and Jensen method was chosen due to its simplicity and its ability to provide reliable sensitivity in detecting antimalarial activity (Trager and Jensen, 1976).

MATERIAL AND METHODS

Method of research

The transformation of eugenol was performed through a Mannich reaction using formaldehyde and aniline as starting materials, which yielded iminium salts. These iminium salts were then treated with eugenol. The reflux process runs for 4, 6, and 10 hours, after which we perform product screening using Gas Chromatography-Mass Spectrometry (GC-MS). GC-MS analysis was performed using a Shimadzu GC-MS-QP2010 system equipped with a GC-2010 gas chromatograph. Separation was carried out on an HP-5 capillary column (30 m × 0.25 mm i.d., 0.25 µm film

thickness; Agilent Technologies, USA) with a non-polar stationary phase consisting of 5% phenyl-95% dimethylpolysiloxane. The column oven temperature was programmed as follows: an initial temperature of 70°C was maintained for 2 minutes, then increased at a rate of 10°C/min to 305°C and held for 25 minutes. The injection temperature was set at 310°C, and the injection mode was splitless with a split ratio of 80:1. The sampling time was 1.0 minutes. Acetylene was used as the carrier gas at a pressure of 19.8 kPa, with a column flow rate of 0.55 mL/min, a total flow rate of 47.9 mL/min, a purge flow rate of 3.0 mL/min, and a linear velocity of 27.3 cm/s. For MS detection, the ion source temperature was set at 250°C, and the interface temperature at 300°C. The mass spectrometer was operated in scan mode from m/z 40 to 600, with a scan speed of 1250, an event time of 0.5 seconds, and a solvent cut time of 3.5 minutes. Data acquisition was conducted over a period of 4.0 to 30.0 minutes.

The purification process utilizes column chromatography with a stationary phase of silica gel 60 and a mobile phase of hexane: ethyl acetate (10:1). The structures of the compounds were confirmed by FTIR and ^1H NMR spectroscopy, and the compounds were subjected to an *in silico* study for ADMET screening using the pkCSM application. We also conducted molecular docking and molecular dynamics evaluation of plasmepsin (PDB ID: 1LEE) using Molexus ver. 7 and MOE software. *In vitro* antimalarial test with the

Trager and Jensen method. The research scheme is shown in Fig. 2.

Synthesis procedure

Eugenol (0.5 g, 3.04 mmol) was placed into a round-bottom flask and dissolved in 6.0 mL of methanol. Subsequently, aniline (0.84 mL, 9.14 mmol) and formalin (1.0 mL, 12.18 mmol) were added to the flask. The reaction mixture was stirred at room temperature for 15 minutes, followed by refluxing at 70°C for 4, 6, and 10 hours. Reaction progress was monitored by thin-layer chromatography (TLC) using ethyl acetate: n-hexane (2:5) as the mobile phase, observed under UV light at a wavelength of 254 nm (Putra et al., 2016)

After reflux, each reaction product was diluted to a concentration of 100 µg/mL in ethyl acetate and analysed by GC-MS. A sample of 10–20 µL was injected, vaporized at 310°C, and passed through an HP-5 column using acetylene as the carrier gas (Putra et al, 2024b).

The purification process involved liquid-liquid extraction. The reaction mixture was extracted with distilled water and ethyl acetate three times. The ethyl acetate phase was washed with a saturated NaCl solution to remove residual water and then separated using a separatory funnel. Anhydrous Na_2SO_4 was added to the ethyl acetate phase to absorb any remaining water. The Na_2SO_4 was subsequently removed by filtration through filter paper (Putra et al., 2016).

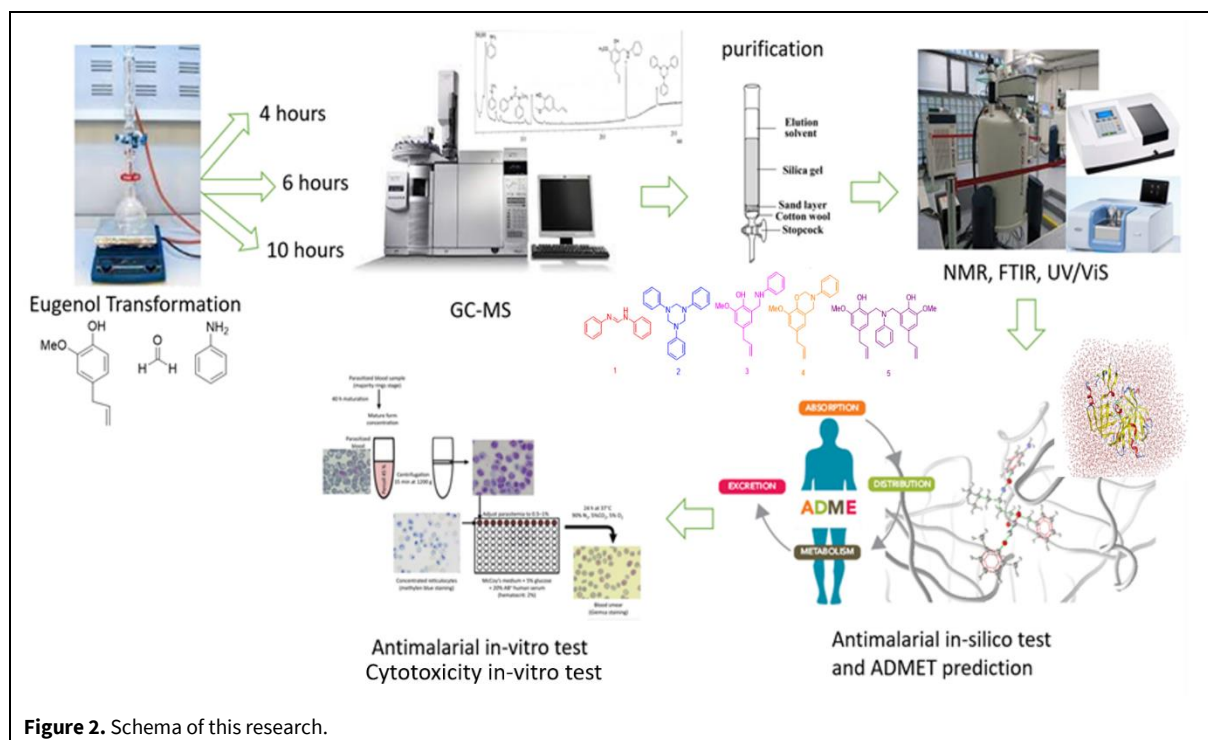


Figure 2. Schema of this research.

Purification was continued with column chromatography, using silica gel as the stationary phase and a mobile phase of n-hexane:ethyl acetate (10:1), yielding a pure isolate. The isolate was then evaluated for yield calculation, followed by qualitative testing and structural characterization using FTIR and H-NMR analysis (Putra et al., 2016).

In silico procedure

Molecular docking test

Chloroquine and five compounds resulting from eugenol transformation via the Mannich reaction were prepared as ligands by designing them in 2D using ChemBioDraw version 11 software. The 2D structures were then converted into 3D structures using the molecular operating environment (MOE) software. Each 3D ligand was subjected to a minimal energy calculation using the MMFF94x force field, and the optimized ligands were saved as PDB files (Putra et al., 2017b; 2023; Thomas, 1996).

Plasmepsin II enzyme was obtained from the Protein Data Bank (PDB) with PDB ID: 1LEE. The enzyme structure was prepared using Molexus Ver. 7 software (Asojo et al., 2002). Validation of the docking protocol was performed by redocking the native ligand with plasmepsin II, ensuring that the Molexus version was accurate for docking chloroquine and the five eugenol transformation compounds.

Molecular dynamics test

Molecular dynamics simulations were performed on six compounds, including the native ligand chloroquine and five eugenol transformation compounds, using MOE software. Simulations were run for 100 ns to study the stability of ligand-receptor interactions with plasmepsin II (PDB ID: 1LEE). The simulation employed the Amber 14:EHT force field under normal pressure temperature (NPT) conditions. Key parameters for the simulation included a temperature of 300 K, a pressure of 1.01325 bar, water solvent box dimensions of 1 Å × 1 Å × 1 Å, an NPT ensemble with periodic boundary conditions, Recording intervals of every 10 ns, a checkpoint interval of 500 steps, and a time step of 0.002 ns. All systems were well-minimized and equilibrated before being subjected to the 100-ns MD simulation. The force field parameters were obtained from OPLS-AA (Guo et al., 2010; Kesuma et al., 2025; Kumar et al., 2020; Murthy et al., 2018; Ruswanto et al., 2022).

Pharmacokinetics/absorption, distribution, metabolism, excretion, and toxicity (ADMET) prediction

The pharmacokinetic profiles of the compounds were predicted using online tools. The compounds were evaluated for the Lipinski Rule of Five using SCF Bio (<http://www.scfbio-uitd.res.in/software/drugdesign/lipinski.jsp>). Data for ADMET were obtained using pkCSM (<https://biosig.lab.uq.edu.au/pkcsm/prediction>) and SwissADME (<http://www.swissadme.ch/index.php>) (Daina et al., 2017; Jayaraam et al., 2013; Pires et al., 2015; Suhud et al., 2019).

To predict elimination/excretion parameters such as the elimination constant (k) and half-life ($t_{1/2}$), the total clearance (Cl) and volume of distribution (Vd) values obtained from pkCSM were used. The equations [1-3] are as follows (Kesuma et al., 2025; Shargel and Yu, 2019; Sulistyowaty et al., 2025).

$$Cl = k \cdot Vd \quad [1]$$

Where Cl = Total Clearance, k = Elimination rate constant, Vd = Volume of distribution.

The elimination rate constant (k) was calculated by the mean of equation [2].

$$k = \frac{Cl}{Vd} \quad [2]$$

Most drugs follow first-order elimination kinetics, allowing the half-life ($t_{1/2}$) to be calculated as equation [3].

$$t_{1/2} = \frac{\ln 2}{k} \quad [3]$$

Where $t_{1/2}$ = Half-life, $\ln 2 = 0.693$, k = Elimination rate constant

Antimalarial in vitro assay

Plasmodium falciparum strain 3D7, a chloroquine-sensitive strain, was obtained from the Malaria Laboratory, Centre for Natural Product Medicine Research and Development, Institute of Tropical Diseases, Universitas Airlangga (UNAIR), Surabaya, Indonesia. The culture was maintained using a modified version of the Trager and Jensen method (Trager and Jensen, 1976). Parasites were cultured in fresh human O-positive red blood cells (RBCs) at a 5% haematocrit in complete RPMI-1640 medium (Sigma), supplemented with 10% (v/v) human O-positive serum, 25 mM HEPES buffer (Applichem Panreac), 50 µg/mL hypoxanthine (Sigma), 2 mg/mL NaHCO₃, and 10 µg/mL gentamycin (Sigma). Incubation was carried out at 37°C in a modified candle jar.

Human RBCs and serum were supplied by the Indonesian Red Cross, Surabaya, Indonesia, approved

under ethical clearance certificate No. 856-KE. The culture medium was refreshed daily, and parasitaemia was kept below 5% for routine subculturing. Parasitaemia levels were monitored by examining thin blood smears stained with Giemsa. Parasite cultures were synchronized using 5% (w/v) D-sorbitol, as described by Lambros and Vanderberg (1979).

An *in vitro* antimalarial activity assay was conducted using a blood medium with a parasitaemia level of approximately $\pm 1\%$ and a haematocrit of 5%. *P. falciparum* strain 3D7 was incubated for 48 hours. Ten milligrams of the sample were diluted in 1 mL of dimethyl sulfoxide (DMSO). The sample was then further diluted in RPMI-1640 medium and used to prepare serial dilutions. Each test compound and chloroquine, used as a reference drug standard, were prepared in five concentrations: 0.01 $\mu\text{g/mL}$, 0.1 $\mu\text{g/mL}$, 1 $\mu\text{g/mL}$, 10 $\mu\text{g/mL}$, and 100 $\mu\text{g/mL}$, and incubated for 48 hours. Thin blood smears were prepared from the culture, fixed with methanol for 5 minutes, stained with 10% Giemsa stain for 30 minutes, rinsed with water, and air-dried. The percentage of parasite growth inhibition was calculated using the following equation [4].

Data from the antimalarial activity tests for each treatment were analysed using probit analysis in SPSS software to determine the IC_{50} values, and a sigmoid dose-response curve was plotted using GraphPad Prism. The antimalarial activity assay was performed in triplicate (Hidayati et al., 2023)

Cytotoxic *in vitro* assay

Sterilization of equipment and materials was performed prior to conducting the *in vitro* cytotoxicity assay on the Vero cell line (ATCC CRL-1587). Vero cells originate from the normal epithelial cells of the kidney of the African green monkey (*Chlorocebus aethiops*). The initial step involved thawing the cells previously stored in liquid nitrogen (-70°C). The cryopreserved cells were thawed in a water bath until the temperature reached 37°C . Subsequently, the cell suspension was transferred into a 15 mL centrifuge tube, and phosphate-buffered saline (PBS) was added to a final volume of 10 mL. The suspension was centrifuged at 1200 rpm for 10 minutes. After discarding the supernatant, the cell pellet was resuspended in 10 mL of complete growth medium and transferred into a 10 cm Petri dish. The cells were then incubated overnight in a CO_2 incubator at 37°C with 5% CO_2 (Kesuma et al., 2022)

Upon reaching sufficient confluency, the cells were subjected to trypsinization. The growth medium was discarded, and the cells were washed three times with 5 mL of PBS (pH 7.4) to remove any residual medium.

Next, 2 mL of 0.1% trypsin was added, and the cells were incubated for 3–5 minutes in the CO_2 incubator. Once detachment occurred, 2 mL of growth medium was added to neutralize the trypsin. The cell suspension was transferred to a 15 mL centrifuge tube, and PBS was added to a final volume of 10 mL. The suspension was centrifuged again at 1200 rpm for 10 minutes. After discarding the supernatant, the cell pellet was resuspended in 2–3 mL of fresh growth medium. The cell concentration was determined using a hemocytometer. Cells were then seeded into a 96-well microplate at a density of 1000 cells/100 μL per well.

Sample preparation and treatment

Compounds exhibiting pharmacological activity, categorized as having excellent antimalarial activity, were dissolved in DMSO to prepare a 1000 $\mu\text{g/mL}$ stock solution. Serial dilutions were performed using growth medium to obtain final concentrations of 500, 250, 125, 62.5, 31.25, and 15.625 $\mu\text{g/mL}$.

The old medium in each well was aspirated, and 100 μL of the test solution was added to each well. Control groups included a cell control (cells + medium without treatment) and a medium control (medium only without cells). The plates were incubated again at 37°C with 5% CO_2 for 24 hours.

MTT assay procedure

After the 24-hour incubation period, the test solutions were discarded, and the cells were rinsed once with PBS. Subsequently, 100 μL of MTT reagent (at a final concentration of 10%) was added to each well, and the plates were incubated for 4 hours at 37°C under 5% CO_2 . Following incubation, 50 μL of a 10% SDS solution was added to each well to solubilize the formazan crystals. The plates were then kept at room temperature in a dark, closed container for 12–24 hours. The absorbance was measured at 590 nm using an ELISA microplate reader. The absorbance values were used to calculate the percentage inhibition of Vero cell growth using the following equation [5].

Data from cytotoxicity *in vitro* assay were analysed using probit analysis in SPSS software to determine the CC_{50} values, and a sigmoid dose-response curve was plotted using GraphPad Prism. Cytotoxicity assay was performed in triplicate.

CC_{50} values are a crucial parameter for calculating the selectivity index (SI), which is a key safety aspect in the development of new drugs (Indrayanto et al., 2021). The SI can be determined using the following equation [6].

$$\text{Parasite Growth Inhibition (\%)} = 100\% - \left(\frac{\% \text{Parasite growth in Sample concentration}}{\% \text{Parasite growth in Placebo}} \right) \times 100\% \quad [4]$$

$$\text{Vero growth inhibition (\%)} = 100\% - \left(\frac{\text{Abs. of treated cells} - \text{Abs. of control media}}{\text{Abs. of control cells line} - \text{Abs. of control media}} \right) \times 100\% \quad [5]$$

$$SI = \left(\frac{\text{Cytotoxicity Concentration}_{50}}{\text{Inhibitory Concentration}_{50}} \right) \quad [6]$$

Statistical analysis

This research involved the synthesis process, molecular docking, *in vitro* antimalarial assays, and cytotoxicity assay on the Vero cell line, each conducted in triplicate ($n = 3$). Result accuracy was determined using the average values, and precision was evaluated by calculating the standard deviation (SD). The IC_{50} and CC_{50} values were calculated using the probit analysis method with a significance level of $p < 0.05$, employing IBM SPSS version 27. All data were analyzed using one-way analysis of variance (ANOVA). If the ANOVA result showed statistical significance ($p < 0.05$), Tukey's Honest Significant Difference (HSD) test was performed as a post hoc test to determine the differences between groups. The data are expressed as mean $\pm$ SD, and the value of $p < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Synthesis and GC-MS, and TLC observation

Eugenol reacted with aniline and formaldehyde under reflux for 4, 6, and 10 hours, and each reaction was observed using GC-MS. The reflux process, conducted for 4 hours, resulted in the formation of compounds **1**, **2**, and **3**, whereas compounds **4** and **5** required a longer reflux duration (Fig. 3).

During the 4-hour reflux process, the starting materials, including eugenol and aniline, remained present. Compounds **1** and **2** formed first due to the reaction mechanism between aniline and formaldehyde (Figs. 4-6). These compounds subsequently reacted with eugenol to form compound **3**, as illustrated in Fig. 7.

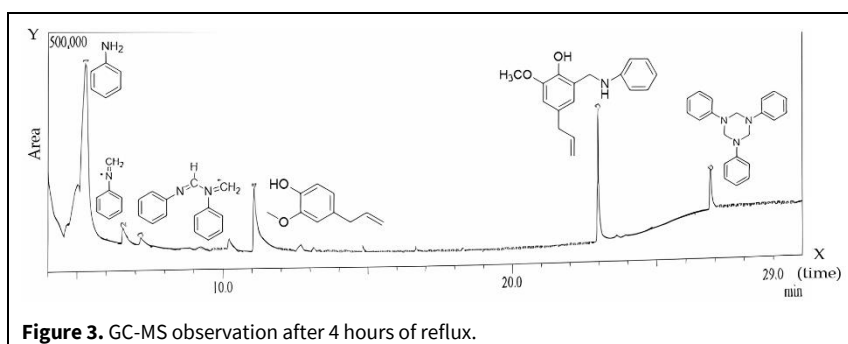


Figure 3. GC-MS observation after 4 hours of reflux.

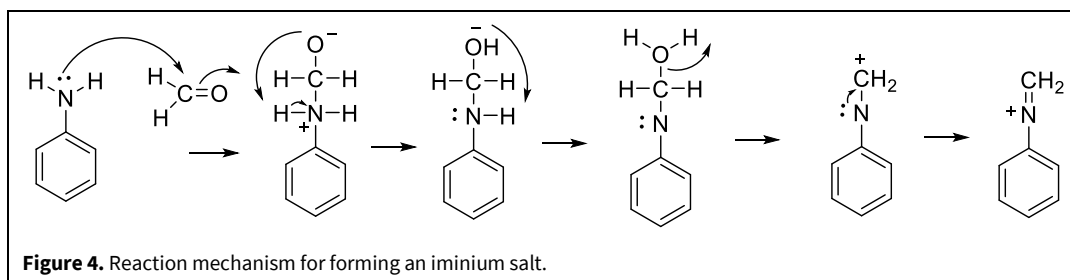


Figure 4. Reaction mechanism for forming an iminium salt.

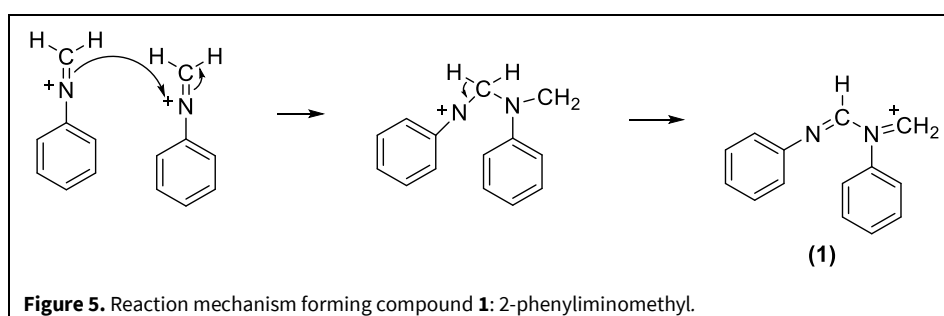
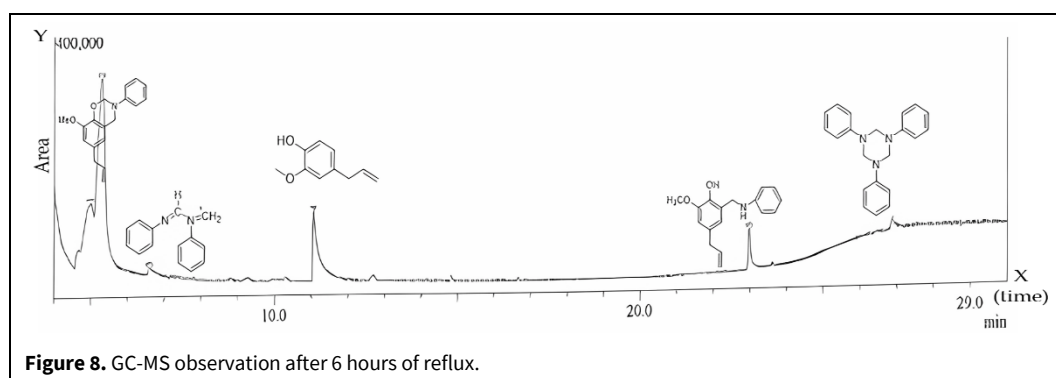
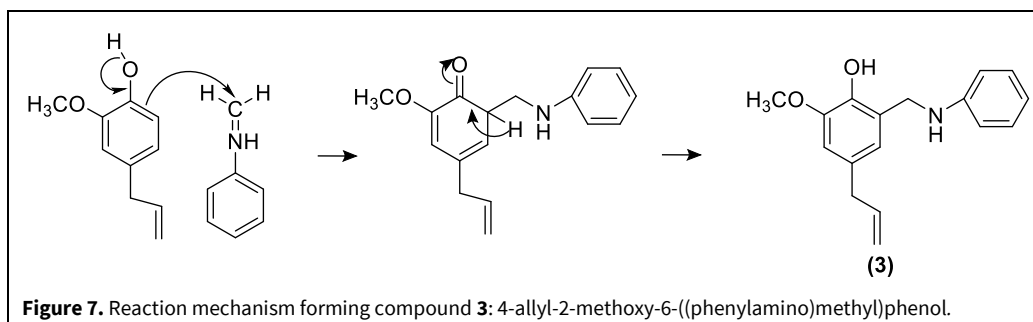
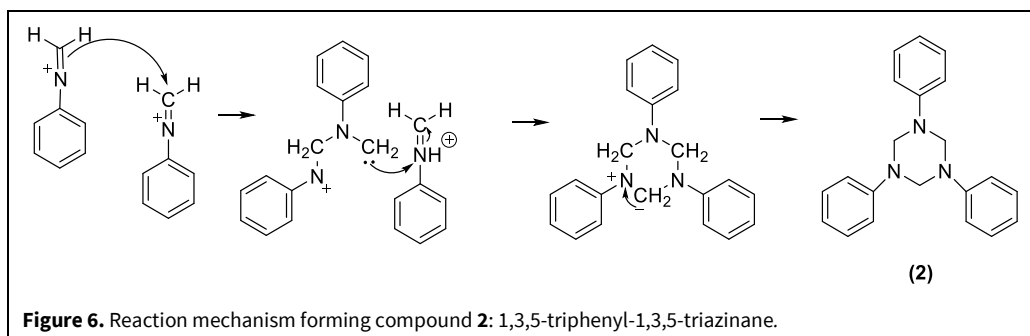


Figure 5. Reaction mechanism forming compound **1**: 2-phenyliminomethyl.



Compound **3** serves as the starting material for synthesizing compounds **4** and **5**. Compound **4** begins to form after a reflux duration of 6 hours, with the quantities of compounds **1** and **2** decreasing as compound **4** becomes more prevalent. This observation is supported by GC-MS data from the 6-hour reflux process (Fig. 8). The reaction mechanism leading to the formation of compound **4** is shown in Fig. 9. The benzoxazine ring is often formed due to intramolecular interactions, leading to a cyclization process (Putra et al., 2017a; Widiyana et al., 2017).

Compound **5** was only obtained after 10 hours of reflux. This result is supported by GC-MS data from the 10-hour reflux process (Fig. 10). The reaction mechanism leading to the formation of compound **5** is illustrated in Fig. 11 (Gondokesumo et al., 2021; Putra et al., 2016).

TLC results using ethyl acetate:n-hexane (2:5) as the mobile phase, observed under UV light at a wavelength of 254 nm, are shown in Fig. 12. Compound **5**

appeared on the TLC plate with an R_f value of 0.62 after 10 hours of reflux.

Results of purification and elucidation

The five synthesized compounds were isolated using a simple column chromatography method. The stationary phase consisted of silica gel 60, while the mobile phase was a mixture of n-hexane and ethyl acetate (10:1). Isolated compounds were analysed using FTIR and H-NMR spectroscopy. Full elucidation of their structures, including FTIR, UV-Vis, H-NMR, C-NMR, and HRMS data, had already been reported in prior research (Putra et al., 2016). The results of the structural confirmation for compounds **1-5**, using FTIR and H-NMR, are summarized below.

Compound 1: (phenyl((phenylimino)methyl)-1*H*-azanylidene)methylum

FT-IR (KBr disk) cm^{-1} : 3431 (-NH), 1621 and 1498 (-C=C- aromatic), 3071(-CH sp^2). $^1\text{H-NMR}$ (400 MHz,

CDCl_3): δ 8.07 (d, 1H, $J=7.8$ Hz), δ 7.99 (d, 1H, $J=7.8$ Hz), δ 7.40 (t, 2H), δ 7.26 (t, 2H), δ 6.83-7.01 (m, 5 H).

Compound 2: 1,3,5-triphenyl-1,3,5-triazinane

FT-IR (KBr disk) cm^{-1} : 3359 (-NH), 1621 and 1467 (-C=C- aromatic), 3036 (-CH sp^2), 1175 (C-N). $^1\text{H-NMR}$ (400 MHz, CDCl_3): δ 7.21-7.26 (m, 6H), δ 6.88-6.94 (m, 9H), δ 3.28 (s, 6H).

Compound 3: 4-allyl-2-methoxy-6-((phenylamino)methyl)phenol

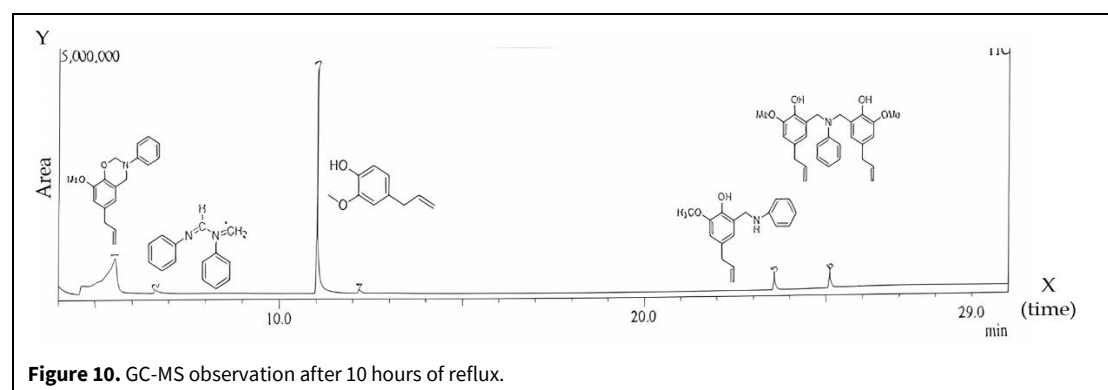
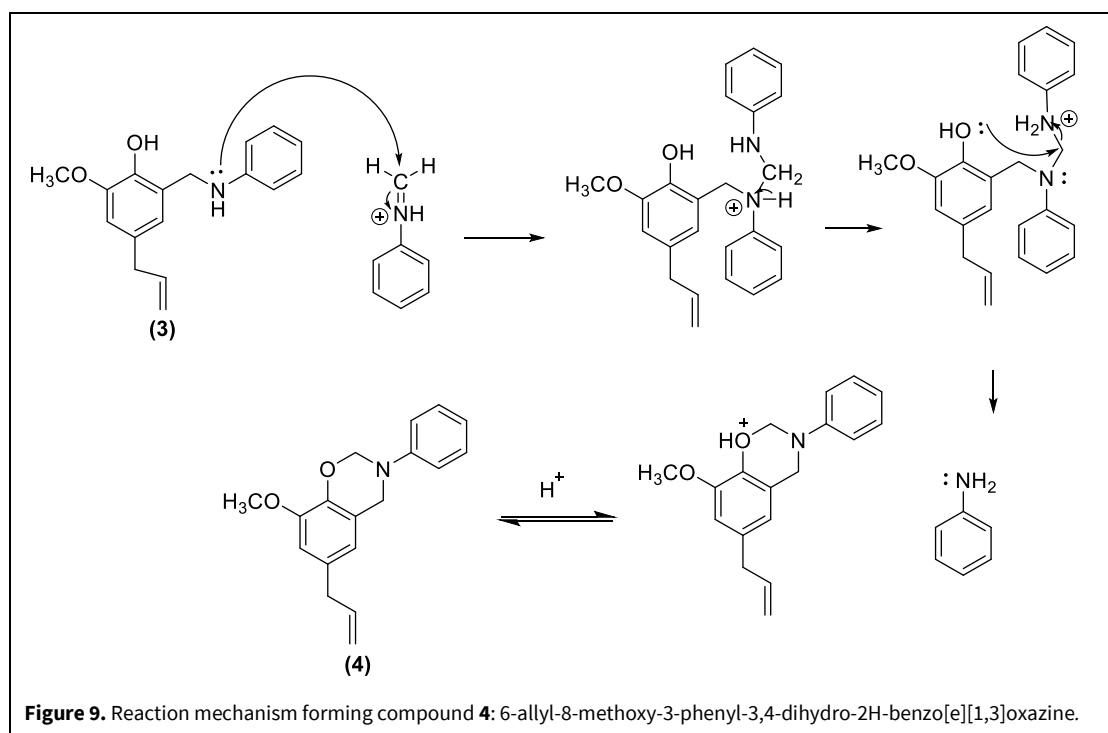
FT-IR (KBr disk) cm^{-1} : 3500 (-OH), 3003 (-N-H), 3076 (-CH sp^2), 2938 (-CH sp^3), 1638 (-C=C- aliphatic); 1604 and 1519 (-C=C- aromatic), 1267 (C-O), 1083 (C-N). $^1\text{H-NMR}$ (400 MHz, CDCl_3): δ 7.15-7.19 (m, 2H), δ 6.70-6.75 (m, 4H), δ 6.62 (s, 1H), δ 5.87-5.97 (m, 1H), δ 4.33 (s, 2H), δ 3.86 (s, 3H), δ 3.28 (d, 2H, $J=6.8$ Hz).

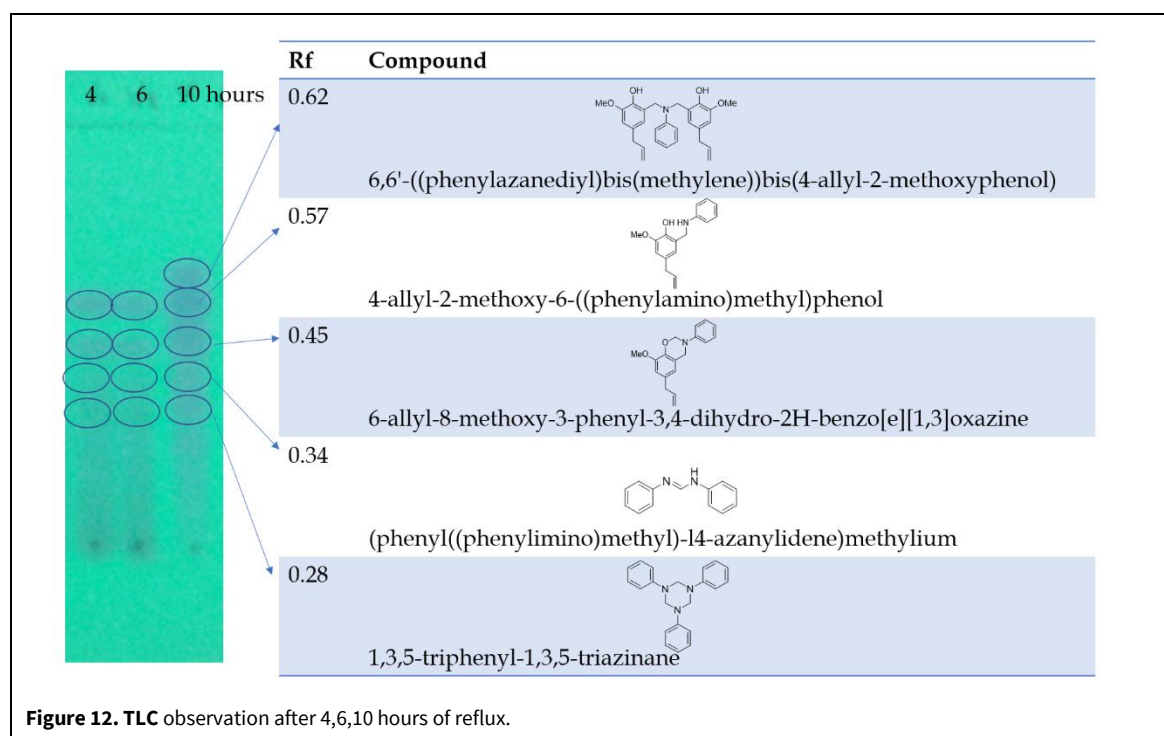
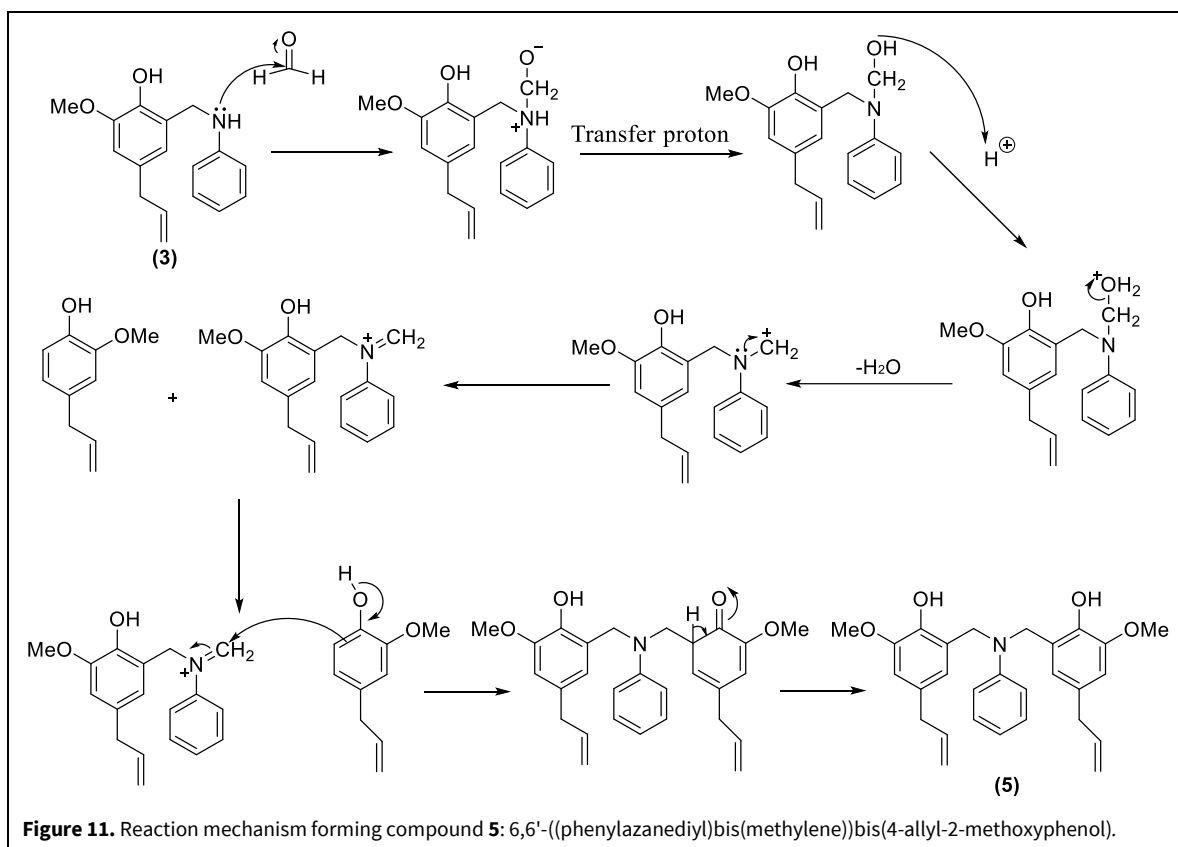
Compound 4: 6-allyl-8-methoxy-3-phenyl-3,4-dihydro-2H-benzo[e][1,3]oxazine

FT-IR (KBr disk) cm^{-1} : 3414 (-OH), 3060 (-CH sp^2), 2902 (-CH sp^3), 1639 (-C=C- aliphatic), 1601 and 1455 (-C=C- aromatic), 1265 (C-O), 1083 (C-N). $^1\text{H-NMR}$ (400 MHz, CDCl_3): δ 7.22-7.26 (m, 2H), δ 7.10-7.12 (m, 2H), δ 6.89-6.93 (m, 1H), δ 6.54 (s, 1H), δ 6.45 (s, 2=1H), 5.87-5.96 (m, 1H), δ 5.41 (s, 2H), δ 5.04-5.10 (m, 2H), δ 4.60 (s, 2H), δ 3.83 (s, 3H), δ 3.28 (d, 2H, $J=6.8$ Hz).

Compound 5: 6,6'-((phenylazanediyl)bis(methylene))bis(4-allyl-2-methoxyphenol)

FT-IR (KBr disk) cm^{-1} : 3500 (-OH), 3003 (-N-H), 3076 (-CH sp^2), 2938 (-CH sp^3), 1638 (-C=C- aliphatic); 1604 and 1519 (-C=C- aromatic), 1267 (C-O), 1083 (C-N). $^1\text{H-NMR}$ (400 MHz, CDCl_3): δ 6.55 (s, 2H), δ 5.83-5.93 (m, 2H), δ 4.98-5.05 (m, 4H), δ 4.47 (s, 4H), δ 3.84 (s, 6H), δ 3.23 (d, 4H, $J=6.8$ Hz).





Result of *in silico* test

Molecular docking result

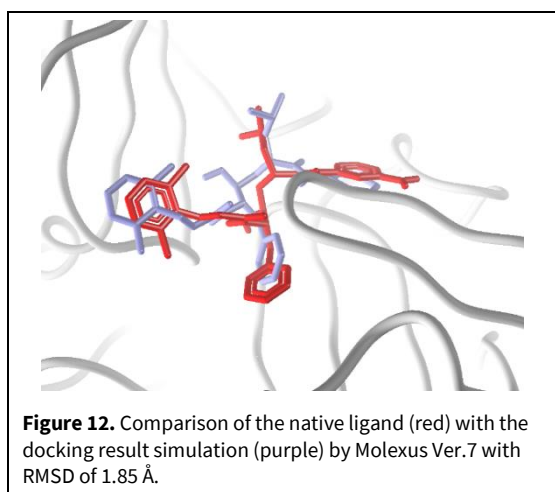
Eugenol's transformation products, the native ligand, and chloroquine were prepared as ligands by

drawing them in 2D in ChemBioDraw version 16 software. 2-Dimensional ligand was then converted into the 3-Dimensional ligand in Chem3D ver.16. 3-D ligand performs the most stable minimal energy calculation by means of the MMFF94 calculation, and the ligand is stored with the PDB file (Thomas, 1996).

The enzyme plasmepsin II was obtained from the Protein Data Bank (PDB) with PDB ID 1LEE, which was subsequently prepared using Molexus ver. 7 (Asojo et al., 2002). Plasmepsin II and its comparison ligand was redocked to validate the Molexus ver.7. Therefore, it could be used to dock eugenol's transformation products (compounds 1-5).

Redocking aims to validate the downloaded PDB files and the software used for the eugenol transformation products (compounds 1-5) method, ensuring that valid and correct results are obtained. The redocking acceptance parameter is the root mean square deviation (RMSD) value $< 2.0 \text{ \AA}$ (Thomsen and Christensen, 2006).

The grid box binding site was set $X = 32.07 \text{ \AA}$; $Y = 33.21 \text{ \AA}$; $Z = 12.51 \text{ \AA}$ with a cavity surrounded by 27 amino acids, they are Ile 32; Asp 34; Gly 36; Ser 37; Ala 38; Met 75; Asn 76; Tyr 77; Val 78; Ser 79; Phe 111; Phe 120; Ile 123; Leu 131; Ile 133; Tyr 192; Ile 212; Asp 214; Gly 216; Thr 217; Ser 218; Ala 219; Thr 221; Ile 290; Leu 292; Phe 294; Ile 300. The result of the redocking validation process with RMSD $1.85 \text{ \AA}$ is shown in Fig. 12. Hence, these results indicate that the method is valid for the docking test of the tested compound since the RMSD obtained is less than $2 \text{ \AA}$ (Putra et al., 2023; Thomsen and Christensen, 2006). The RMSD value indicates the compatibility of the ligand coordinates from the crystallographic results with those of the ligand coordinates that were redocked to the native ligand, achieving a distance of $1.85 \text{ \AA}$, which aligns with the docking process criteria.



Based on the docking results, a moldock score was obtained, representing the predicted interaction strength between the drug and receptor. A lower score indicates a higher degree of compatibility between the ligand and receptor, suggesting stronger interactions (Thomsen and Christensen, 2006). The docking results can also be visualized and interpreted to provide insights into ligand-receptor interactions, including

hydrogen bonding, hydrophobic interactions, and electronic interactions, as presented in Table 1 and Fig. 13.

Native ligand demonstrated the lowest moldock score (-157.57 kcal/mol) on plasmepsin compared to chloroquine and the eugenol transformation products (compounds 1-5). Interaction of native ligand with plasmepsin included hydrogen bonds with distances $< 3 \text{ \AA}$ involving the $-\text{OH}$ group of native ligand at two amino acids: Asp 214 ($\text{C}=\text{O}$) and Thr 217 ($\text{C}=\text{O}$), and the $-\text{NH}$ group of native ligand at amino acid Leu 131 ($\text{C}=\text{O}$). Hydrogen bonds with distances $> 3 \text{ \AA}$ involved $-\text{NH}$ group of native ligand at two amino acids: Gly 36 ($\text{C}=\text{O}$) and Gly 216 ($\text{C}=\text{O}$). Steric interactions occurred with 6 amino acids: Gly 36, Tyr 77, Val 78, Asp 214, Ser 218, and Ala 219.

Chloroquine had a moldock score of -96.56 kcal/mol , with 4 steric interactions involving amino acids Asp 34, Gly 36, Tyr 77, and Asp 214. Based on the moldock scores ranked from lowest to highest energy, the order was as follows: Compound 5 $<$ compound 2 $<$ chloroquine $<$ compound 3 = compound 4 $<$ compound 1.

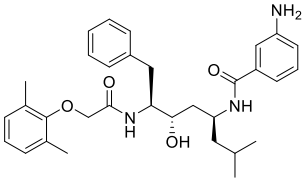
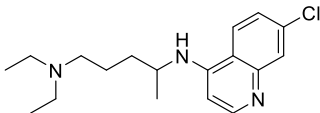
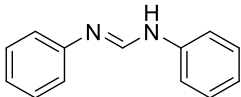
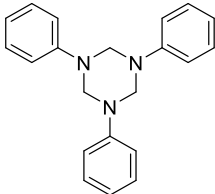
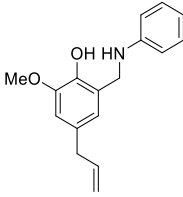
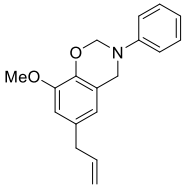
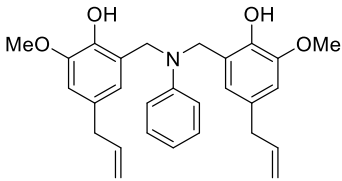
Among the compounds, compound 5 showed the strongest potential to inhibit plasmepsin due to its lowest moldock score. Compound 5 exhibited a hydrogen bond between its $-\text{OH}$ group and amino acid Gly 36 ($\text{C}=\text{O}$), Asp 214 ($-\text{OH}$) to $\text{C}=\text{O}$ from compound 5 and steric interactions with Asp 214 and Gly 216.

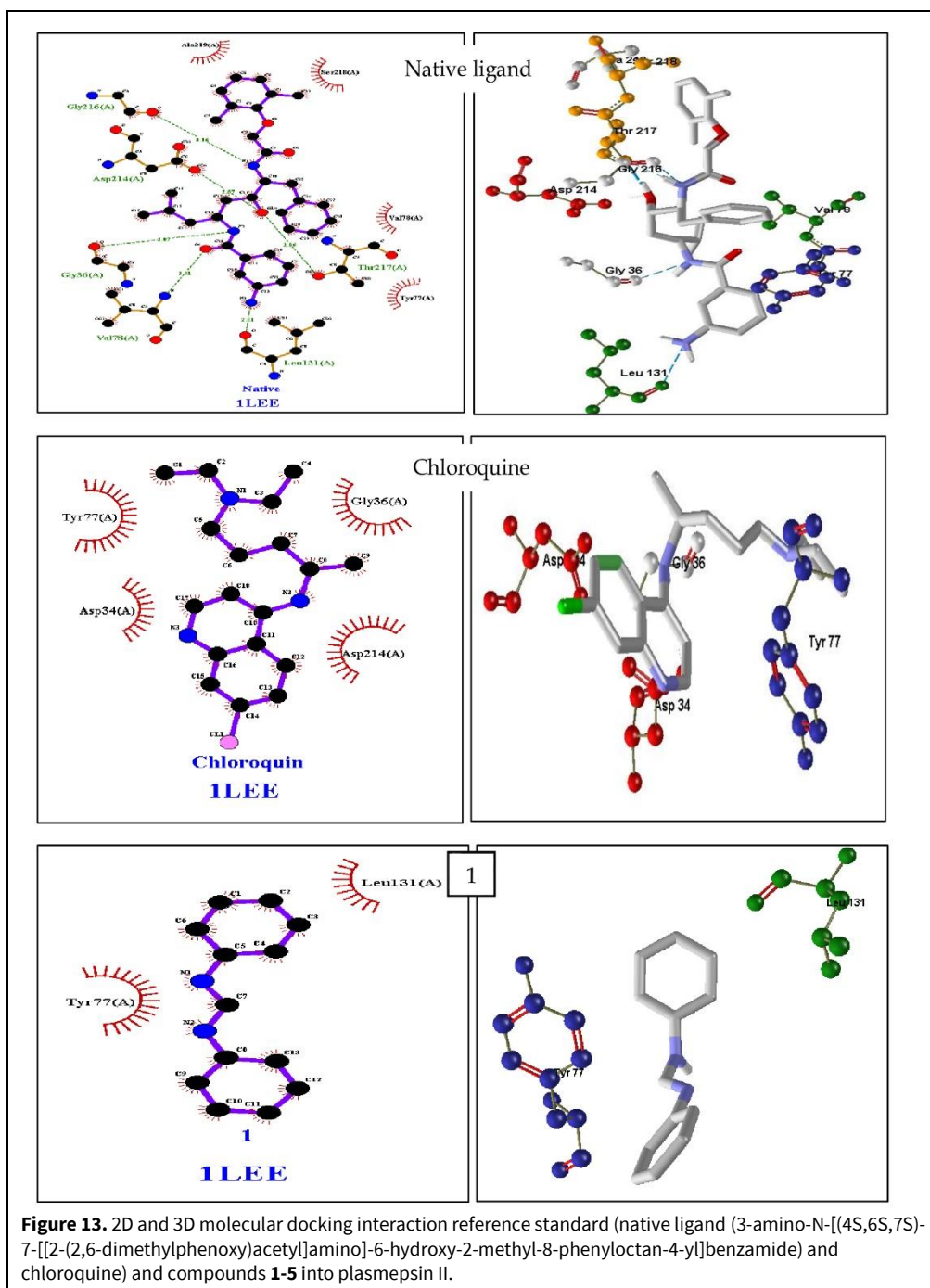
Molecular dynamics results

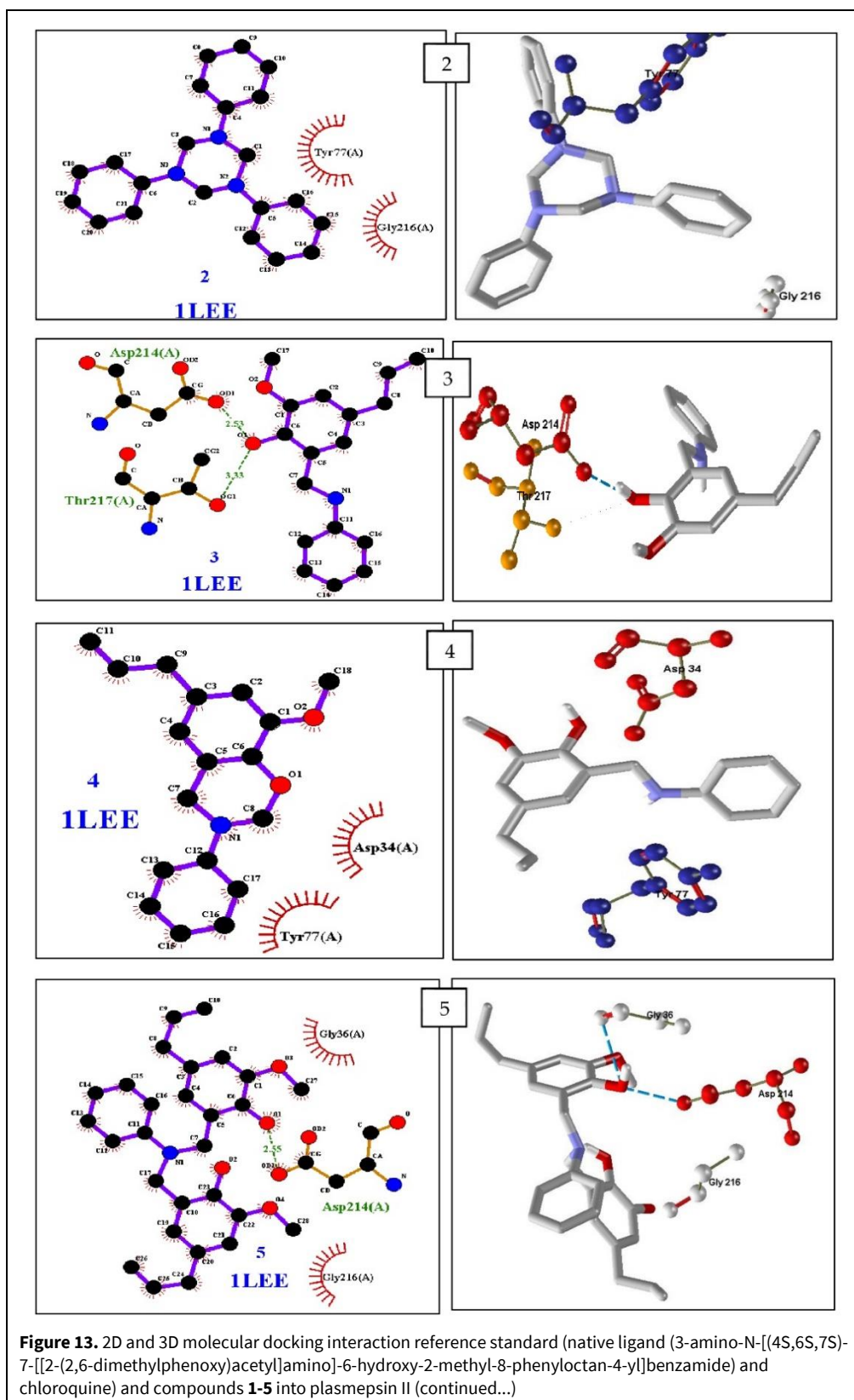
Molecular docking results were further supported by molecular dynamics simulations conducted over 100 ns. Native ligand exhibited the lowest average RMSD value of $3.544 \text{ \AA}$, which falls into a relatively stable category for ligand-receptor complex stability ($3\text{--}5 \text{ \AA}$) (Ekowati et al., 2023; Hollingsworth and Dror, 2018). Compound 5 demonstrated an average RMSD of $4.454 \text{ \AA}$, indicating a relatively more stable ligand-receptor interaction compared to reference drug chloroquine, which showed a higher average RMSD of $5.575 \text{ \AA}$, as illustrated in Fig. 14.

In terms of ligand-receptor complex stability, the ranking from most to least stable is as follows: native ([3-amino-N-\[\(4S,6S,7S\)-7-\[\[2-\(2,6-dimethylphenoxy\)acetyl\]amino\]-6-hydroxy-2-methyl-8-phenyloctan-4-yl\]benzamide](#)) $>$ compound 5 $>$ chloroquine $>$ compound 4 $>$ compound 3 $>$ compound 2 $>$ compound 1, as shown in Fig. 14. Molecular dynamics findings were consistent with the docking results, in which the native ligand also had the lowest binding energy (-157.57 kcal/mol), followed by compound 5 (-127.98 kcal/mol), chloroquine (-96.56 kcal/mol), and the other compounds, as presented in Table 1.

Table 1. Molecular docking result of native ligand, chloroquine, and eugenol's transformation products (compounds 1-5) into the active site of plasmepsin II.

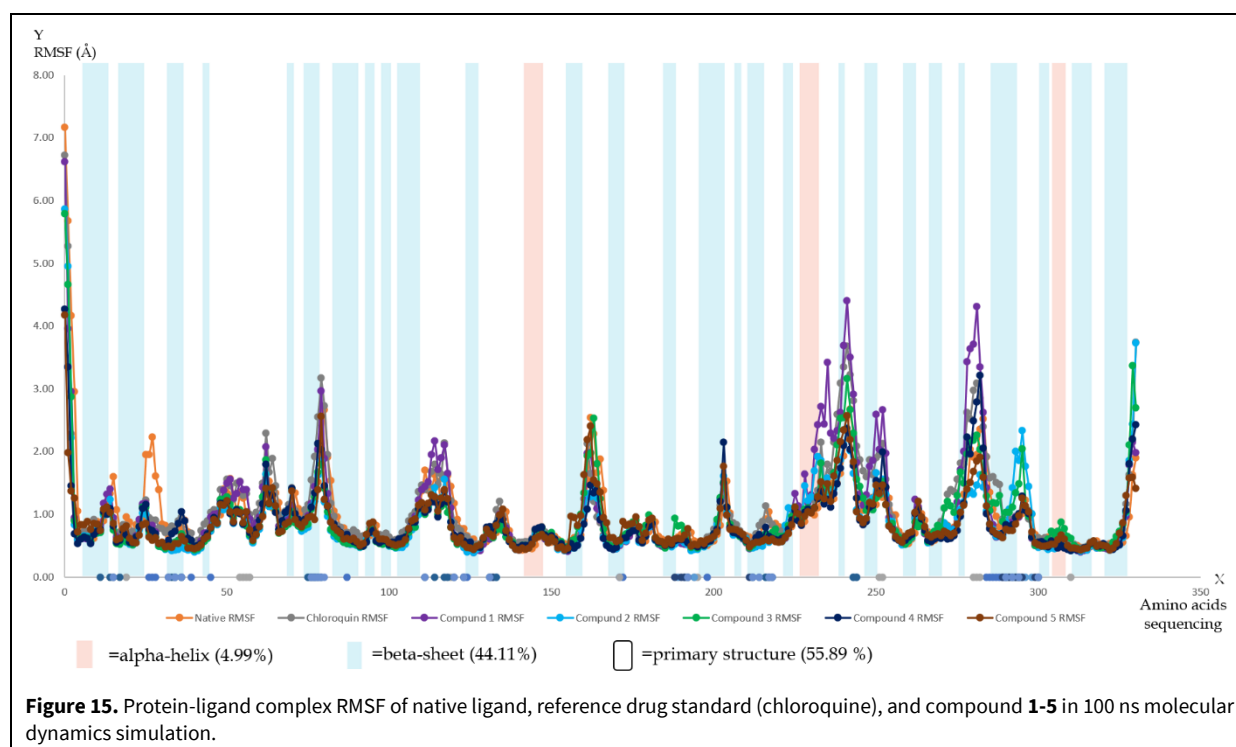
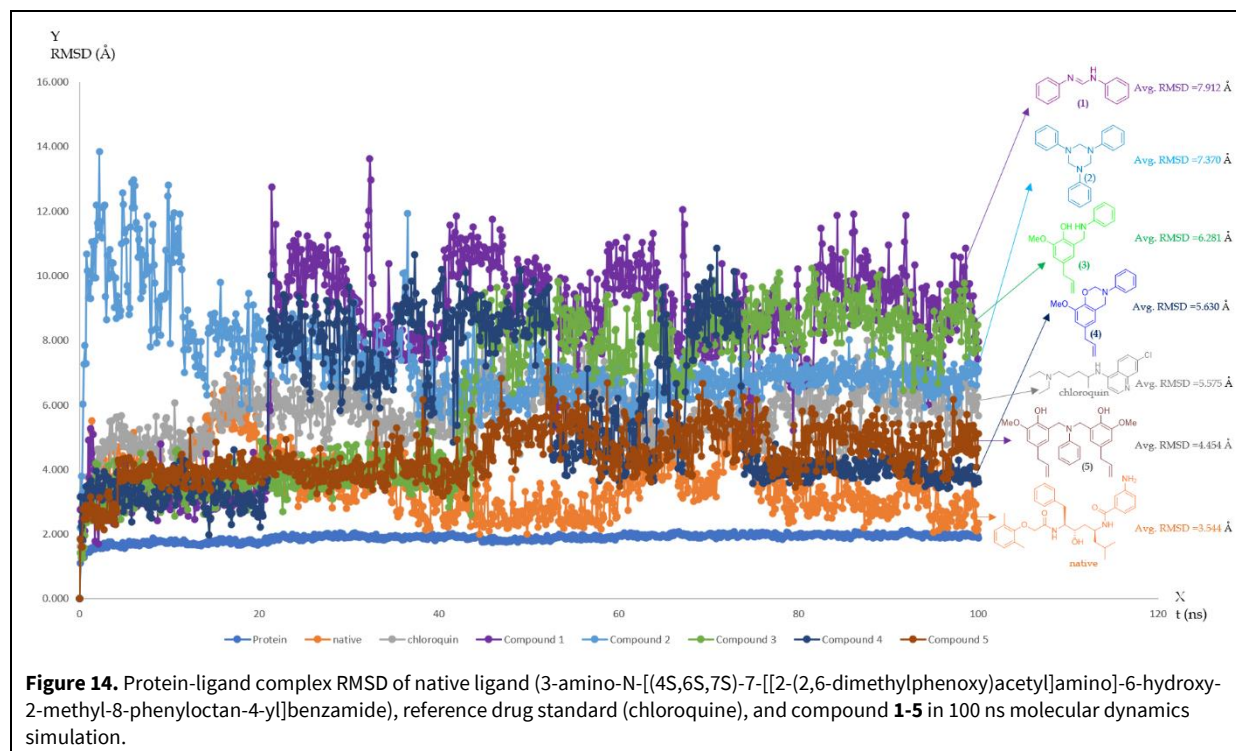
Compound	Moldock score (kcal/mol)	Hydrogen bond	Steric interaction
 Native ligand (3-amino-N-[(4S,6S,7S)-7-[[2-(2,6-dimethylphenoxy)acetyl]amino]-6-hydroxy-2-methyl-8-phenyloctan-4-yl]benzamide)	-157.57 ± 2.86	Gly 36 (3.07 Å) Val 78 (3.31 Å) Leu 131 (2.01 Å) Asp 214 (2.57 Å) Gly 216 (3.16 Å) Thr 217 (2.86 Å)	Gly 36 Tyr 77 Val 78 Asp 214 Ser 218 Ala 219
 Chloroquine	-96.56 ± 0.58	-	Asp 34 Gly 36 Tyr 77 Asp 214
 Compound 1	-77.92 ± 0.24	-	Tyr 77 Leu 131
 Compound 2	-99.44 ± 0.98	-	Tyr 77 Gly 216
 Compound 3	-90.63 ± 2.59	Asp 214 (2.53 Å) Thr 217 (3.33 Å)	Asp 214 Thr 217
 Compound 4	-90.54 ± 0.69	-	Asp 36 Tyr 77
 Compound 5	-127.98 ± 1.37	Gly 36 (3.17 Å) Asp 214 (2.55 Å)	Gly 36 Asp 214 Gly 216





Total protein secondary structure elements of plasmeprin II accounted for 44.11%, consisting of 4.99% α -helix and 39.12% β -sheet structures (Fig. 15). The remaining 55.89% was composed of primary structural elements. RMSF analysis revealed that compounds **1-5** interacted with several of the same amino

acid residues as the native ligand, with RMSF values $<1.5 \text{ \AA}$ (Ekowati et al., 2023; Ghahremanian et al., 2022). An RMSF value of $<1.5 \text{ \AA}$ indicates low flexibility, suggesting that these compounds bind precisely at the core domain or active site pocket of plasmeprin II, as illustrated in Fig. 15.



Molecular dynamics results of each compound over a 100 ns simulation demonstrated stable interactions with several amino acid residues, as summarized below:

Native ligand (3-amino-N-[(4S,6S,7S)-7-[[2-(2,6-dimethylphenoxy)acetyl]amino]-6-hydroxy-2-methyl-8-phenyloctan-4-yl]benzamide)

Hydrogen bonds were formed with six amino acid residues: Asp 34 (46.1%; 0.74); Gly 36 (32.1%; 0.91 Å);

Asn 76 (10.2%; 1.25 Å); Val 78 (29.0%; 2.06 Å); Ser 79 (19.1%; 2.66 Å); Leu 131 (21.0%; 0.81 Å).

Ionic/electronic interactions were formed with two amino acid residues: Asp 34 (24.0%; 0.74 Å); Asp 214 (36.1%; 0.80 Å).

Hydrophobic/steric interactions were formed with 18 amino acid residues: Met 15 (0.1%); Ile 32 (1.1%); Met 75 (0.1%); Tyr 77 (80.0%); Val 78 (22.2%); Phe 111 (57.2%); Ala 117 (0.1%); Phe 120 (36.6%); Ile 123 (16.7%); Ile 133 (0.5%); Tyr 192 (0.8%); Ala 219 (0.3%); Pro 243 (0.1%); Phe 244 (0.4%); Ile 290 (0.8%); Leu 292 (1.3%); Phe 294 (0.5%); Ile 300 (0.5%).

Water bridges interactions were formed with 19 amino acid residues: Asp 34 (42.5%); Gly 36 (12.2%); Ser 37 (0.1%); Ala 38 (18.3%); Asn 39 (20.1%); Glu 74 (0.1%); Asn 76 (22.2%); Tyr 77 (5.1%); Val 78 (47.2%); Ser 79 (12.1%); Thr 114 (8.2%); Leu 131 (10.2%); Tyr 192 (7.0%); Asp 214 (13.1%); Gly 216 (8.2%); Thr 217 (22.2%); Ser (0.1%).

Protein-ligand contact results for the native ligand are shown in Fig. 16.

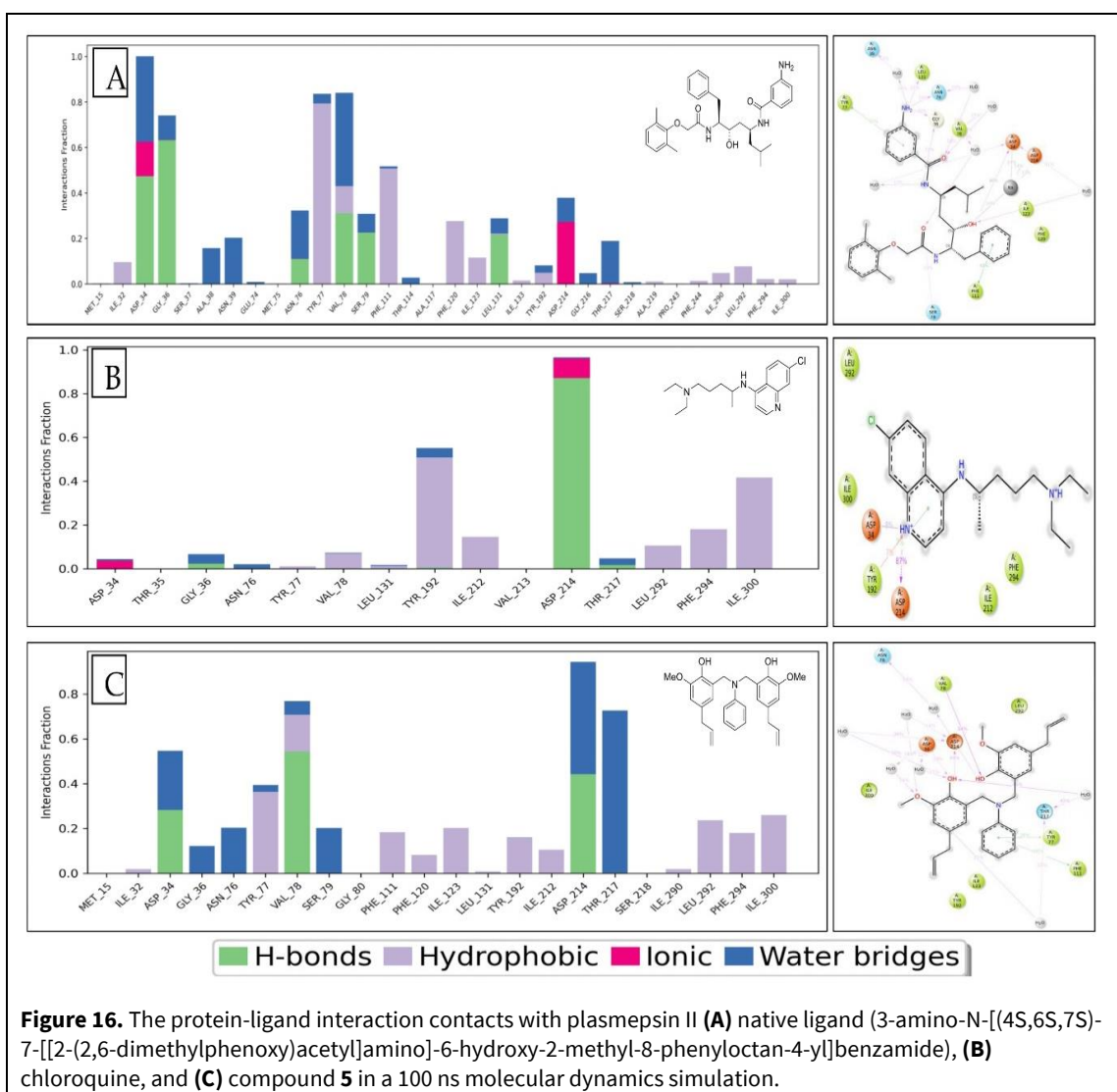
Chloroquine

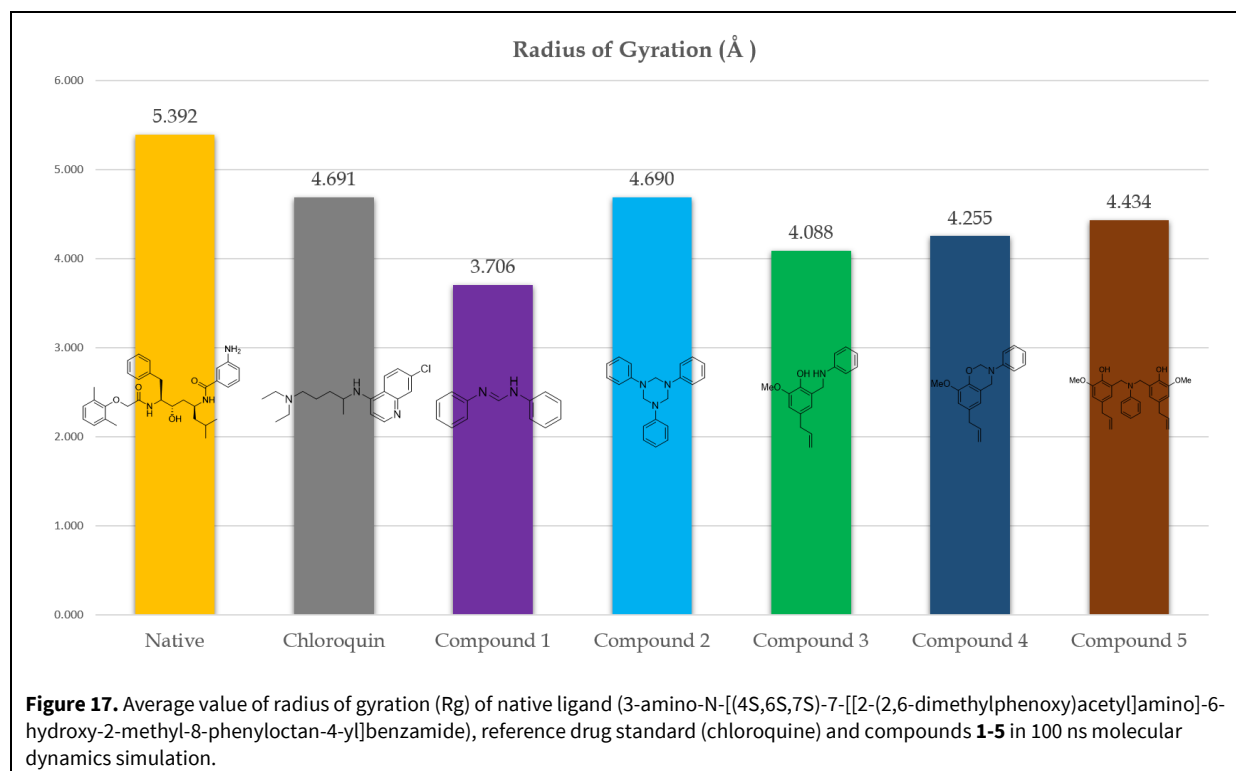
Hydrogen bonds were formed with four amino acid residues: Gly 36 (2.3%, 0.95 Å); Thr 192 (0.5%, 0.58 Å); Asp 214 (87.1%, 0.82 Å); Thr 217 (8.71%, 0.91 Å).

Hydrophobic/steric interactions were formed with 6 amino acid residues: Thr 77 (1.1%; 2.55 Å); Leu 131 (0.71%; 1.06 Å); Ile 212 (14.6%; 0.57 Å); Leu 292 (10.6%; 0.89 Å); Phe 294 (18.1%; 0.96 Å); Ile 300 (41.8%; 0.59 Å).

Ionic/electronic interactions were formed with two amino acid residues: Asp 34 (3.7%; 0.64 Å); Asp 214 (9.0%; 0.82 Å).

Water bridges interactions were formed with seven amino acid residues: Thr 35 (0.1%); Gly 36 (4.4%); Asn 76 (2.1%); Leu 131 (0.1%); Tyr 192 (4.3%); Val 213 (0.1%); Thr 217 (3.1%). Protein-ligand contact results for chloroquine are presented in Fig. 16.





Compound 5

hydrogen bonds were formed with four amino acid residues: Asp 34 (28.3%; 0.53 Å); Val 78 (54.3%; 2.56 Å); Asp 214 (44.3%; 0.58 Å); Thr 217 (0.3%; 0.60 Å).

Hydrophobic/steric interactions were formed with 14 amino acid residues: Met 15 (0.1%; 0.84 Å); Tyr 77 (20.3%; 1.39 Å); Val 78 (16.5%; 2.56 Å); Phe 111 (18.4%; 1.18 Å); Phe 120 (8.2%; 0.55 Å); Ile 123 (20.3%; 0.47 Å); Leu 131 (0.2%; 0.65 Å); Tyr 192 (15.9%; 0.49 Å); Ile 212 (15.9%; 0.56 Å); Ile 290 (2.0%; 0.74 Å); Leu 292 (23.7%; 0.85 Å); Phe 294 (18.0%; 0.98 Å); Ile 300 (26.1%; 0.49 Å).

Water bridges interactions were formed with 10 amino acid residues: Asp (34%); Gly 36 (12.2%); Asn 76 (20.3%); Tyr 77 (3.1%); Ser 79 (54.3%); Gly 80 (0.1%); Asp (50 %); Thr (72.3%). Protein-ligand contact results for compound **5** are shown in Fig. 16.

Radius of gyration (Rg) is a key parameter for evaluating the structural compactness and conformational stability of a protein-ligand complex during molecular dynamics simulations. In this research, the Rg values of the native ligand and chloroquine were found to be 5.392 Å and 4.691 Å, respectively. Compounds **1-4** exhibited lower Rg values compared to both the native ligand and the reference drug chloroquine, indicating that these five compounds demonstrated better structural compactness and conformational stability of the protein-ligand complex, as shown in Fig. 17.

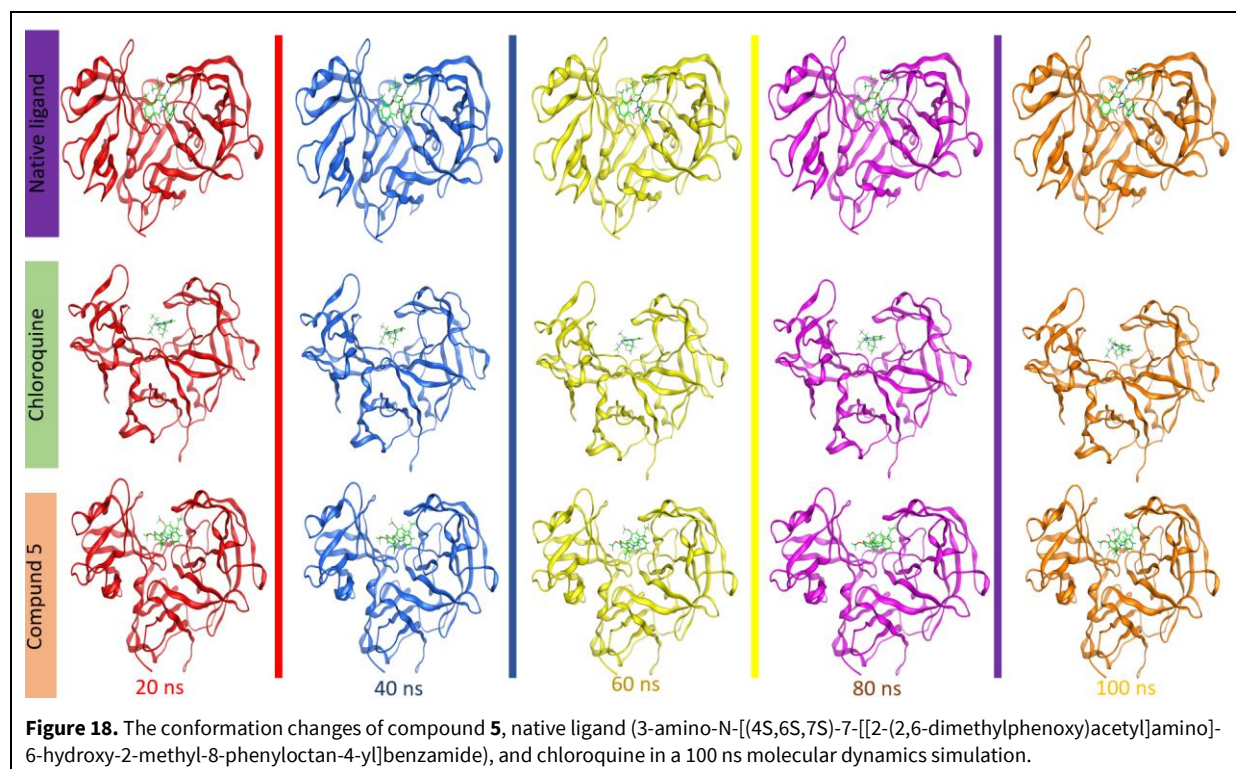
The results from molecular docking were supported by molecular dynamics simulations, which ran

from 0 to 100 ns. The native ligand, chloroquine, and compound **5** exhibited stable binding interactions, as evidenced by their ability to remain within the active site of plasmepsin II throughout the simulation, as demonstrated by the trajectory results in Fig. 18.

Pharmacokinetics prediction result

Based on the ADMET prediction results using SCFbio software, compounds **1-5** comply with Lipinski's Rule of Five criteria, which include Log P < 5, hydrogen bond donors (HBD) ≤ 5, hydrogen bond acceptors (HBA) ≤ 10, molar refractivity (MR) within the range of 40-130 Å, and molecular weight (Mr) ≤ 500. This compliance suggests that compounds **1-5** are predicted to be absorbed effectively through the gastrointestinal tract (GIT) (Jayaram et al., 2013; Putra et al., 2024a; Sulistyowaty et al., 2021). Similarly, chloroquine also fulfils Lipinski's Rule of Five, supporting its formulation as an oral tablet for peroral administration.

The absorption prediction for compounds **1-5**, based on their Caco-2 permeability values, indicates that compounds **1-4** demonstrate better permeability compared to chloroquine. However, compound **5** has a permeability = 2.61×10^{-6} cm/s, categorizing it as having low permeability. Caco-2 cell lines, derived from human epithelial colorectal adenocarcinoma cells, are widely used as *in vitro* models for human intestinal mucosa to predict the permeability of compounds intended for oral drug absorption.



According to Pires et al. (2015), compounds with a Caco-2 permeability value $>8 \times 10^{-6}$ cm/s are categorized as having high permeability. In addition to permeability, water solubility is a crucial parameter for predicting absorption in the GIT. Compound **1** has a $\log S = -3.12$, categorizing it as soluble. Compounds **2** and **3** have $\log S$ values ranging from -5.72 to -4.53 , placing them in the moderately soluble category, similar to chloroquine, which has a $\log S = -4.55$. On the other hand, compound **5** has a $\log S = -6.32$, classifying it as poorly soluble (Daina et al., 2017; Putra et al., 2024a).

Based on the Biopharmaceutical Classification System (BCS), new compounds **1-4** and chloroquine are categorized under BCS class I, characterized by high permeability and high solubility. Consequently, the percentage of human intestinal absorption for these compounds is predicted to be excellent or very good ($>80\%$). Meanwhile, compound **5** is categorized under BCS class IV, characterized by low permeability and low solubility. As a result, the predicted percentage of human intestinal absorption for compound **5** is poor ($<30\%$) (Daina et al., 2017; Pires et al., 2015; Putra et al., 2024; Suhud et al., 2019).

P-glycoprotein (P-gp) is an active membrane transporter belonging to the ATP-binding cassette (ABC) family, which is located on the cell membrane and is predominantly found in the GIT. P-gp functions to efflux exogenous compounds (drugs) from the cell using energy derived from ATP (Trevor et al., 2015). There are two types of P-gp inhibitors: P-gp I inhibitors and

P-gp II inhibitors (Pires et al., 2015). P-gp I inhibitors play a more significant role compared to P-gp II inhibitors. P-gp I inhibitors can markedly increase the intracellular concentration of P-gp substrates, thereby enhancing absorption and bioavailability, which may ultimately lead to a higher risk of toxic effects compared to P-gp II inhibitors (Pires et al., 2015). Compounds that act as P-gp I inhibitors also have a higher potential to cause drug–drug, herbal–drug, or food–drug interactions. Based on prediction results, compounds **1**, **3**, **4**, and chloroquine do not inhibit P-gp I activity, indicating a relatively low risk of interaction at the absorption phase with other drugs, herbal products, or food. In contrast, compounds **2** and **5** were predicted to inhibit P-gp I activity, and therefore, these two compounds require careful consideration regarding the potential for drug–drug, herbal–drug, or food–drug interactions.

The volume of distribution (Vd) is a theoretical parameter indicating the extent to which a drug is distributed between plasma and tissues. A large Vd indicates a greater concentration of the drug in tissues relative to plasma, whereas a small Vd suggests a higher concentration in plasma than in tissues. A small Vd is defined as <0.71 L/kg, a large Vd as >2.81 L/kg, and values between 0.71 – 2.81 L/kg fall into the moderate distribution category (Pires et al., 2015).

The predicted distribution for compound **4** (4.645 L/kg) and chloroquine (22.284 L/kg) places them in the large volume of distribution category, indicating a higher concentration in tissues. Compounds **1-3** and **5** have Vd values ranging from 0.824 to 2.716 L/kg,

categorizing them as having moderate volume of distribution.

Cytochrome P450 (CYP) is a crucial enzyme involved in detoxification, primarily located in the liver (Shargel and Yu, 2019; Trevor et al., 2015). Many drugs are either deactivated or activated by this enzyme. Therefore, it is crucial to predict whether drug compounds inhibit cytochrome P450. Some isoforms of CYP include CYP2D6 and CYP3A4, which together metabolize more than 90% of drugs. Predicting this metabolism is essential for screening whether new compounds are likely to interact with other drugs during the metabolic phase. In addition to CYP2D6 and CYP3A4, there are also CYP1A2, CYP2C9, and CYP2C19 isoforms. If these enzymes are inhibited, it can increase the concentration of free drugs in plasma, leading to toxic conditions. Thus, predicting the metabolism phase of new compounds under development is necessary to anticipate potential drug interactions during this phase. Some drugs that may have increased levels due to the inhibition of CYP2D6 include beta-blockers, lidocaine, antiarrhythmics, opioids, clozapine, and antidepressants. Drugs affected by CYP3A4 inhibitors include antidepressants, azole antifungals, benzodiazepines, calcium channel blockers (CCBs), proton pump inhibitors (PPIs), SSRIs, HIV protease inhibitors, antiarrhythmics, and HMG-CoA reductase inhibitors. Drugs impacted by CYP1A2 inhibitors include acetaminophen, clozapine, haloperidol, theophylline, and tricyclic antidepressants (TCAs). Drugs affected by CYP2C9 inhibitors include steroids, doxorubicin, phenytoin, barbiturates, celecoxib, ibuprofen, and chloramphenicol. Lastly, drugs influenced by CYP2C19 inhibitors include diazepam, phenytoin, and topiramate (Trevor et al., 2015).

The metabolism prediction results for compounds 1-5 indicate that compound 2 inhibits CYP enzymes the least. Compound 2 only inhibits CYP1A2 and CYP2C19. In contrast, compounds 1 and 5 inhibit CYP3A4, which is the primary enzyme involved in drug metabolism, suggesting that these compounds are likely to interact with a wide range of drugs. Additionally, compound 1 inhibits CYP1A2 and CYP2C19, while compound 5 inhibits CYP2C9 and CYP2C19.

Total clearance is the rate at which a drug is eliminated from the body, encompassing both hepatic clearance (liver metabolism and biliary clearance) and renal clearance (Shargel and Yu, 2019). Total clearance is directly proportional to the Vd and the elimination constant (k), as expressed by the formula $Cl = Vd \cdot k$ (Shargel and Yu, 2019). Consequently, the elimination constant k is directly proportional to clearance (Cl) and inversely proportional to Vd. A higher k value results in a shorter half-life ($t_{1/2}$) of a compound, indicating that the bioavailability of the compound in the body is

reduced more quickly as it is excreted. Conversely, a lower k value leads to a longer ($t_{1/2}$), meaning the bioavailability of the compound remains longer before excretion.

The classification of drug half-life can vary depending on the literature and clinical context. Here are some common classifications based on half-life. A $t_{1/2} < 1$ hour is categorized as ultra short half-life, typically for drugs that require frequent administration, often in the form of IV drips. A $t_{1/2} = 1-4$ hours falls into the short half-life category, for drugs that usually require multiple doses per day to maintain therapeutic levels in the blood. A $t_{1/2} = 4-12$ hours is classified as an intermediate half-life, for drugs generally administered 1-2 times a day to maintain therapeutic levels. A $t_{1/2} = 12-24$ hours is categorized as a long half-life, typically for drugs that need to be administered once daily to maintain therapeutic levels. A $t_{1/2} > 24$ hours is categorized as ultra long half-life, for drugs that typically require administration once a day or every few days to maintain therapeutic levels. Based on the prediction of excretion, compounds 1 and 3 are categorized as intermediate half-life, compound 2 as long half-life, compound 4 as ultra-long half-life, and compound 5 as short half-life.

The toxicity prediction indicates that compounds 1-4 are not hepatotoxic, whereas compound 5 and chloroquine are hepatotoxic. Among them, compound 5 exhibits the highest toxicity, based on its oral rat acute toxicity (ORAT) value, with an LD_{50} of 2.163 mol/kg, making it more toxic than chloroquine and the other compounds. Overall, the pharmacokinetic or ADMET predictions for new compounds 1-3 were conducted using pkCSM software and the SIB online tool. The summarized results are presented in Table 2.

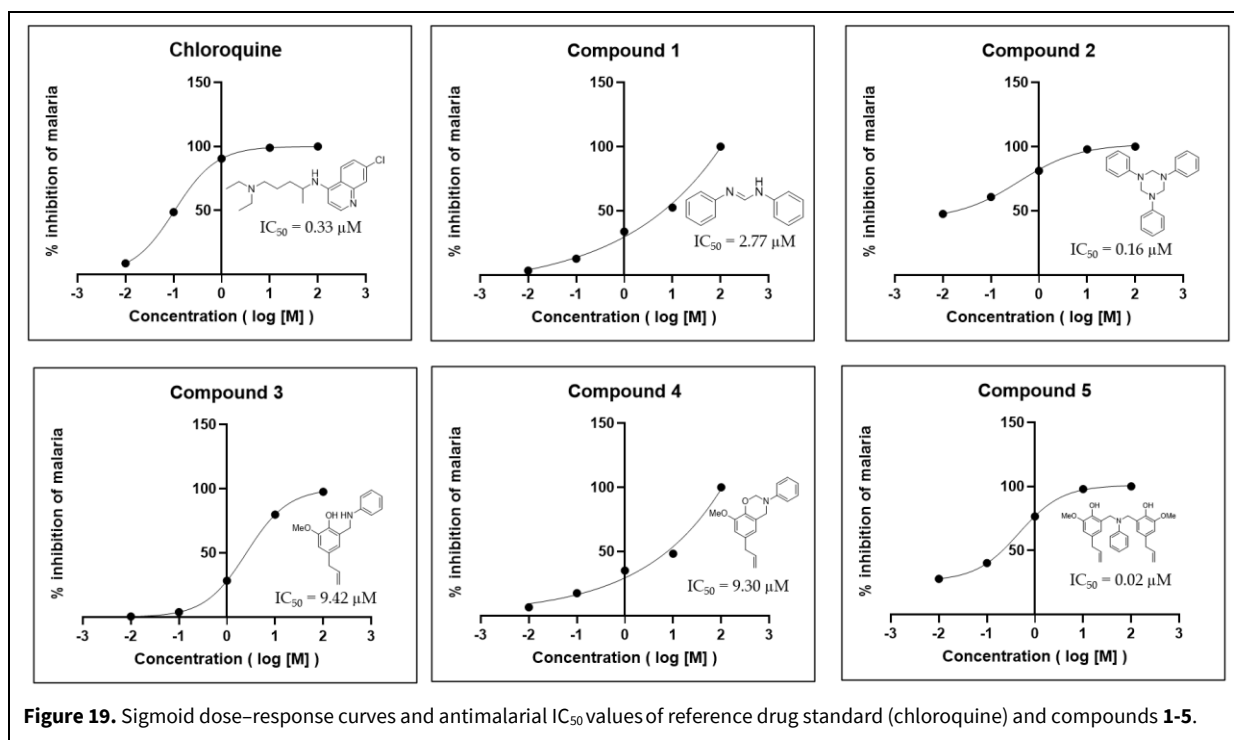
Result of antimalarial and cytotoxicity *in vitro* assay

There are several *in vitro* tests for evaluating antimalarial activity, including the observation of parasite development in blood cells through thick films, isotopic assays, parasite protein quantification, DNA dye intercalation, the Trager and Jensen method, and the Semiautomated Microdilution Technique. A method with good sensitivity and simplicity is the Trager and Jensen method. In the Trager and Jensen method, testing is conducted on parasite cultures with a parasitaemia level of approximately 1% and a haematocrit of 5%, with experimental replication. *P. falciparum* is incubated for two days in blood medium, and thin blood smears are prepared using 10% Giemsa staining. The smears are left for 30 minutes, rinsed with water, and then dried. The percentage of parasitaemia, the percentage of *P. falciparum* growth, and inhibition are calculated through microscopic observation to determine

Table 2. The result of ADMET predictions.

Compound	Absorption			Distribution	Metabolism (CYP inhibitor)					Excretion			Toxicity	
	Caco2 Perm. 10 ⁻⁶ cm/s	Log S	P-gp I Inh.		Vd (L/kg)	2D6	3A4	1A2	2C9	2C19	Total Cl (L/hour/kg)	k (1/hour)	t ½ (hour)	ORAT mol/kg (LD ₅₀)
1	51.17	-3.12	No	1.324	No	Yes	Yes	No	Yes	0.149	0.1127	6.15	2.434	No
2	41.98	-5.72	Yes	2.716	No	No	Yes	No	Yes	0.084	0.0301	22.36	2.221	No
3	46.77	-4.67	No	2.094	No	No	Yes	Yes	Yes	0.154	0.0735	9.43	2.460	No
4	36.22	-4.53	No	4.645	No	No	Yes	Yes	Yes	0.125	0.0269	25.8	2.289	No
5	2.61	-6.32	Yes	0.824	No	Yes	No	Yes	Yes	0.231	0.2806	2.47	2.164	Yes
CQ	30.13	-4.55	No	22.284	Yes	No	No	No	No	0.742	0.0333	20.81	2.766	Yes

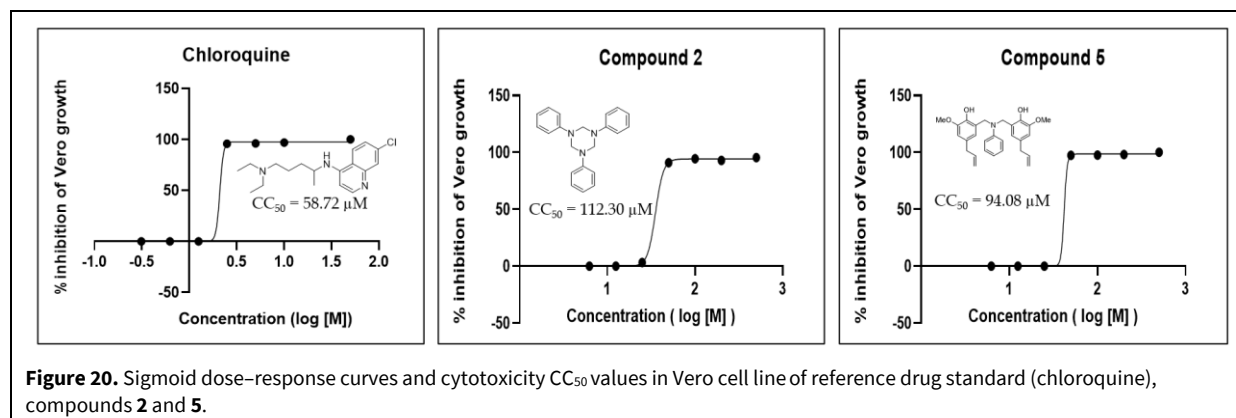
Perm.: Permeability; CQ: Chloroquine; P-gp: P-glycoprotein; Inh: Inhibitor; CYP: Cytochrome P450; Cl: Clearance; k: Elimination constant; t_{1/2}: Half-life; LD₅₀: Lethal dose.

**Figure 19.** Sigmoid dose–response curves and antimalarial IC₅₀ values of reference drug standard (chloroquine) and compounds **1–5**.

the IC₅₀ value. The IC₅₀ results for compounds **1–5** and chloroquine are presented in Fig. 19.

In vitro antimalarial assay using the Trager and Jensen method with *P. falciparum* strain 3D7, which is sensitive to chloroquine, showed that chloroquine has an IC₅₀ = 0.33 μM, indicating excellent antimalarial activity. Compound **5** has an IC₅₀ of 0.02 μM, and compound **2** has an IC₅₀ of 0.16 μM, indicating that they exhibit more potent antimalarial activity compared to chloroquine, as their IC₅₀ values are lower. Both compounds **5** and **2** were categorized as having excellent

antimalarial activity. On the other hand, compounds **1**, **3**, and **4** are categorized as having good antimalarial activity because their IC₅₀ values fall within the range of 1–20 μM (Indrayanto et al., 2021). Recent studies have also reported that eugenol transformation products hold strong potential for development as antimalarial agents (Syahri et al., 2023). The results of one-way ANOVA analysis with a significance level of *p* < 0.05 showed that the IC₅₀ values of compounds **2** and **5** were not significantly different from the IC₅₀ value of chloroquine, indicating that all three compounds fall into the category of excellent antimalarial activity.



In vitro cytotoxicity assays on normal cell lines are essential for evaluating the safety profile of test compounds. Compounds that are cytotoxic to normal cells typically exhibit low CC_{50} values. The Vero cell line is commonly used for cytotoxicity screening because most compounds are excreted through the kidneys. Therefore, damage or death of normal kidney cells upon exposure to the compound serves as an early indicator of potential nephrotoxicity (Indrayanto et al., 2021). In this research, cytotoxicity assays were performed selectively on compounds **2** and **5**, as these compounds demonstrated excellent antimalarial activity. Reference standard drug, chloroquine, was also included in the cytotoxicity assay on Vero cells for comparative purposes. The CC_{50} values of chloroquine, compounds **2** and **5** were presented in Fig. 20. The CC_{50} values for compounds **2** (112.30 μ M) and **5** (94.08 μ M) were higher than the reference drug chloroquine (58.72 μ M), suggesting that compounds **2** and **5** exhibit lower nephrotoxic potential on normal kidney cell lines compared to chloroquine.

Selectivity index (SI) is defined as the ratio of CC_{50} to IC_{50} . If CC_{50} : IC_{50} ratio is below 1, the compound is considered highly toxic and carries a significant risk if advanced to the preclinical testing phase (Indrayanto et al., 2021). Conversely, if the ratio is greater than 10, the compound is categorized as non-toxic or very safe and is eligible for further evaluation in preclinical studies (Indrayanto et al., 2021). SI values for compounds **2**, **5**, and the reference standard drug chloroquine are presented in Table 3. Compound **2** exhibited an SI value = 701.88, compound **5** had an SI = 4074, and chloroquine had an SI = 177.94. All three values exceed the threshold of 10, indicating that these compounds fall within the very safe category, are not nephrotoxic, and are suitable for progression to *in vivo* preclinical trials to further evaluate their antimalarial activity.

In vitro results align with the *in silico* findings, which show that compounds **2** and **5** have lower

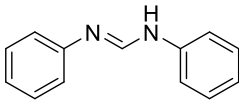
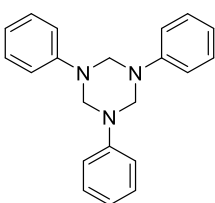
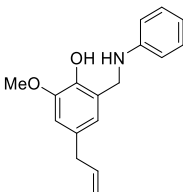
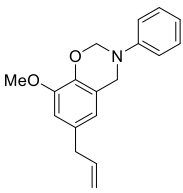
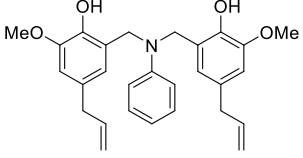
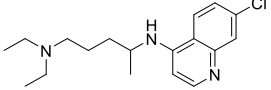
docking energy scores compared to chloroquine. Molecular dynamics results, which spanned from 0 to 100 ns, indicate that chloroquine, along with compounds **2** and **5**, maintain good binding stability as it remains within the active site of plasmeprin. Although compound **5** has the lowest IC_{50} value compared to the others, it should be considered that the pharmacokinetic profile of compound **5** is not as favourable as that of compound **2**. Compound **5** is predicted to have poor absorption and is classified as a BCS class IV compound, characterized by low permeability and solubility. Additionally, in the metabolism phase, compound **5** is predicted to inhibit the CYP 3A4, CYP 2C9, and CYP 2C19 enzymes, suggesting significant potential for drug interactions, especially since CYP 3A4 is the primary enzyme responsible for metabolizing nearly 90% of drug compounds. In the excretion phase, compound **5** has a half-life ($t_{1/2}$) of 2.47 hours, placing it in the category of compounds with short half-lives. In terms of toxicity, compound **5** is predicted to be hepatotoxic, with the lowest LD_{50} value compared to the other test compounds. On the other hand, compound **2** exhibits a more favorable pharmacokinetic profile than compound **5**.

Overall, the transformation products of eugenol can be further developed as antimalarial agents. Future research should focus on cytotoxic assays involving normal hepatic and kidney cell lines.

CONCLUSION

Compounds **5** (6,6'-((phenylazanediy)bis(methylene))bis(4-allyl-2-methoxyphenol)) and **2** (1,3,5-triphenyl-1,3,5-triazine) have IC_{50} values of less than 1 μ M, which categorize them as having excellent activity. These compounds show potential for further investigation as candidates for antimalarial agents.

Table 3. Result of selectivity index (SI) $CC_{50}:IC_{50}$ ratio of compounds **2**, **5**, and chloroquine.

Compound	IC_{50} (μM)	CC_{50} (μM)	SI
 1	2.77 ± 0.89	-	-
 2	0.16 ± 0.03	112.30 ± 1.57	701.88
 3	9.42 ± 0.24	-	-
 4	9.30 ± 0.57	-	-
 5	0.02 ± 0.01	94.08 ± 1.89	4704
 Chloroquine	0.33 ± 0.24	58.72 ± 2.65	177.94

CONFLICT OF INTEREST

The research was conducted entirely free from any involvement in commercial, financial, or personal matters that might be perceived as a possible conflict of interest or that could have influenced the outcomes or interpretation of the study.

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GENERATIVE ARTIFICIAL INTELLIGENCE (AI)

The authors used ChatGPT and Grammarly (IA tools) to improve the language and readability of the manuscript. The authors reviewed and edited the content and take full responsibility for its accuracy. No images were generated or manipulated using AI tools.

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AUTHOR CONTRIBUTION:

Contribution	Putra GS	Sulistyowaty MI	Yahmin Y	Sumari S	Retnosari R	Marfu'ah S	Anwari F	Suhud F	Kesuma D	Ma'arif B	Mardianto R	Sukkaew A	Yamauchi T
Concepts or ideas	x						x						
Design	x						x						
Definition of intellectual content	x				x		x						
Literature search	x	x	x	x	x	x	x	x	x	x	x	x	x
Experimental studies	x									x			
Data acquisition	x	x			x				x			x	
Data analysis	x	x										x	
Statistical analysis	x	x						x	x				
Manuscript preparation	x		x	x	x			x					
Manuscript editing	x							x					x
Manuscript review	x	x	x	x	x	x	x	x	x				

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https://doi.org/10.56499/jppres24.2286_13.s1.347

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