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RESEARCH ARTICLE

Analysis of Alkaloids Compounds and Antimalarial Activity of *Coleus amboinicus* Leaves from Indonesia

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Abstract

Malaria is still considered a major health problem and has a high number of death cases in developing countries. In addition, it is exacerbated by the emergence of resistance to available malaria drugs that encourages the discovery of potential alternative drugs that do not cause resistance and provide better efficacy. There are ten nitrogen compounds belonging to the alkaloid group contained in the ethanolic extract of *C. amboinicus* leaves that have been identified, studied and correlated with their potential as antimalarials *in vitro* and molecular docking. *In vitro* studies of the ethanolic extract of *C. amboinicus* leaves tested on *P. falciparum* strain 3D7 and FCR3 parasites provided inhibition with activity values (IC_{50}) of 13.176 ± 0.006 µg/mL (SI 38.024 ± 0.005) and 49.683 ± 0.007 µg/mL (SI 10.084 ± 0.001), respectively. The results of molecular docking studies of ten N compounds, compounds (4) and (8), showed better binding affinity to the *falciparum-2* receptor with binding energy (–6.5 kcal/mol) and better than chloroquine (–5.8 kcal/mol) and native ligand (–6.0 kcal/mol). Each compound contained in the ethanolic extract of *C. amboinicus* leaves provides potential antimalarial activity by inhibiting the *falciparum-2* receptor. Based on the research data obtained, it provides more supporting evidence for the traditional use of the ethanolic extract of *C. amboinicus* leaves in traditional malaria treatment, and in the future, it provides hope for its development as a more potential antimalarial agent.

Keywords: Antimalarial assay, *Coleus amboinicus,* Molecular docking, *Plasmodium falciparum*

Introduction

Malaria is a highly inflammatory and oxidative infectious disease caused by protozoan parasites of the genus Plasmodium. It caused 627,000 and 597,000 deaths worldwide in 2020 [1] and 2023 [2]. Almost all regions in various countries in the world are at risk of malaria, and although it can be prevented and cured, it

is in fact one of the highest contributors to cases of health problems and death [3]. Concerns about the increase in malarial cases, drug resistance, and the emergence of malaria parasites with deletions in the histidine-rich protein 2 (HRP2) gene greatly affect the accuracy of rapid malaria diagnostic tests [4]. Therefore, researchers in the health and pharmaceutical fields are strongly encouraged to find and develop antimalarial drugs that are effective, efficient, and have better efficacy. There are 5 species of the genus, including *Plasmodium falciparum* (*P. falciparum*), *P. malariae*, *P. vivax*, *P. ovale*, and *P. knowlesi*. Among the five species, the P. falciparum species is the deadliest strain and contributes to the highest mortality rate [5].

The malaria-causing *P. falciparum* parasite has developed resistance to antifolate drugs such as pyrimethamine, which targets the parasite's enzyme *Plasmodium falciparum* dihydrofolate reductase (*Pf*DHFR). This resistance also occurs with combinations of antifolate and sulfa drugs, so new strategies are needed to create antifolate compounds that remain effective against the *P*fDHFR mutations found in resistant parasites [6]. Health problems caused by malaria encourage the discovery and development of potential drugs, one of which is by utilizing bioactive compounds from natural resources. The utilization of bioactive compounds from natural materials is considered an alternative breakthrough and is important for developing antimalarial drug discoveries. One of the plants that is believed to need further research is the *Coleus amboinicus,* Lour. (*C. amboinicus*) leaves plant [7,8].

C. amboinicus is an herbal plant that has many health benefits [9,10]. The abundant content of bioactive compounds such as flavonoids, alkaloids, phenolics, steroids/triterpenoids, tannins, and essential oils provides various pharmacological activities, including antioxidant, antimicrobial, antidiabetic, antibacterial, antimalarial, anticancer, antitumor, and so on [11-13]. The alkaloid compound group is known as an important phytoconstituent with interesting biological properties. In fact, the first antimalarial drug successfully isolated from the cinchona tree was an alkaloid compound (quinine). Several alkaloid groups, including terpenoid alkaloids, indoles, bisindoles, quinolones, and isoquinolines, have been reported to have promising antimalarial activity [14]. Therefore, this study aims to explore, identify, and analyze the alkaloid compounds of the ethanol extract of *C. amboinicus* leaves, which can be used as a guide for the discovery of potential new drugs for better malaria treatment.

Materials and methods

Research materials

C. amboinicus leaves samples were obtained from Lumban Gurning village, Toba district, North Sumatra Province, located at coordinates 2°29′49.200″N 99°10′12.000″E. The samples were collected in April to May 2023, and further determined by a botanist at the Systematics Laboratory, Faculty of Biology, Gadjah Mada University (number 0220/S.Tb./I/2023) [15]. The chemicals used in this study were distilled water (technical grade, CV. Progo), ethanol (pro analytical grade, Merck), chloroform (pro analytical grade, Merck), methanol (LC-MS grade, Merck), formic acid (LC-MS grade, Merck), ethanol (LC-MS grade, Merck), dimethyl sulfoxide (DMSO, pro-analytical Merck), Whatman filter paper No. 1, Dragendorff reagent (pro analytical grade, Merck), Mayer reagent (pro analytical grade, Merck), Weagner reagent (pro analytical grade, Merck), caffeine (pro analytical grade, Merck), phosphate buffer pH 4.7 (technical grade, CV. Progo), bromocresol green (pro analytical grade, Merck), and Roswell Park Memorial Institute (RPMI) medium (Sigma-Aldrich). Meanwhile the instruments employed in this work were laboratory glassware, magnetic blender (Maspion), rotary vacuum evaporator (Heidolph), ultraviolet-visible spectrophotometer (Shimadzu, UV-1,800), and LC–MS were obtained from Thermo Fisher Scientific (Fair Lawn, USA).

Sample preparation and extraction

The *C. amboinicus* leaves samples used were green, fresh, and free from insect contamination. The samples were cleaned under running water, drained, and dried in a modified stainless steel drying cabinet with heating using a monitored lamp at a temperature of 50 °C. The dried samples were powdered using a magnetic blender to increase the surface area of the sample in contact with the solvent so that the extraction process would be maximized. The samples were extracted with ethanol solvent in a macerator container at room temperature with occasional stirring, and the maceration process was carried out for 2×24 h. After reaching the

desired concentration, the extract was filtered and then the residue was re-macerated using the same solvent. The re-maceration process was repeated twice. The liquid extract obtained was concentrated using a rotary vacuum evaporator at 55 °C with a rotation speed of 90 rpm. The thick ethanol extract of *C. amboinicus* leaves obtained was put into a glass bottle and prepared for further analysis.

Screening and determination of alkaloid content

Phytochemical screening of the alkaloid group was carried out using standard reagents, namely Dragendorff, Mayer, and Weagner reagents, following previous research [16-18]. The determination of total alkaloid content was carried out colorimetrically using a UV-Vis spectrophotometer with caffeine as a standard solution. Caffeine was dissolved in hot distilled water to obtain various concentration variations (25, 50, 75, 100 and 125 µg/mL). Each caffeine concentration was taken in 5 mL and then put into a separating funnel. A 5 mL of phosphate buffer (pH 4.7), 5 mL of bromocresol green (1×10^{-4} M), and 5 mL of chloroform were added to the mixture. The liquid-liquid extraction process with the addition of chloroform was repeated three times. The collected chloroform phase was concentrated using a water bath, and then the concentrated chloroform extract was dissolved with exactly 10 mL of chloroform solvent and measured at a wavelength range of 350 - 700 nm to obtain the maximum wavelength [19,20]. The same steps were taken in determining the total alkaloid content in the ethanolic extract of *C. amboinicus* leaves with a concentration of 1,000 µg/mL. The measurement was carried out with three repetitions, and the total alkaloid content in the sample was expressed in mg caffeine equivalent to per gram of ethanol extract (mg CE/g d.w. ethanolic extract). The standard curve of caffeine measured at the maximum wavelength (415 nm) was obtained, i.e., $y = 0.0006x - 0.0086$ ($R^2 = 0.9784$).

Analysis of alkaloid compound groups using LC-HRMS

Fifty milligrams of the concentrated ethanolic extract of *C. amboinicus* leaves were placed in a 2 mL centrifuge tube, then 1 mL of methanol (LC-MS grade) was added and shaken for 1 min. The sample was then centrifuged at 5,000 rpm for 10 min. Analysis was carried out using liquid chromatography and high-resolution orbitrap spectrometry with reference to previous research methods. The chromatography column used was 100 mm×2.1 mm ID×2.6 µm. The mobile phase used was a mixture of water with 0.1% formic acid (A) and a mixture of ethanol with 0.1% formic acid (B), both solvent mixtures according to LC-MS quality, and a flow rate of 0.3 mL/min. Scanning of the analyzed bioactive compounds was carried out using the full MS/dd-MS^2 acquisition mode in positive or negative polarity/ionization conditions [15].

Evaluation of the antimalarial activity

The antimalarial activity test of the ethanolic extract of *C. amboinicus* leaves was evaluated against *P. falciparum* strain 3D7 and FCR3. *P. falciparum* 3D7 is a sensitive strain to chloroquine, cycloguanil, and pyrimethamine but resistant to sulfadoxine, while strain FCR3 is sensitive to pyrimethamine and sulfadoxine but resistant to chloroquine and cycloguanil drugs. The test was carried out by following the procedure with slight modifications referring to previous studies [21-24]. The samples were dissolved in 10% DMSO and diluted with RPMI media to obtain various concentrations (6.25, 12.5, 25, 50, 100 and 200 µg/mL). The solution of each concentration was taken in 100 µL, then 100 µL of inoculum solution containing *P. falciparum* 3D7 parasites (infected red blood cells), then continued incubation for 72 h at 37 °C in an incubator containing 5% CO_2 gas. The same test was carried out on the *P. falciparum* FCR3 and uninfected red blood cells (normal cells). The number of infected and normal blood cells is at least 1,000. The work was carried out with 3 repetitions.

Molecular docking

The target protein that was determined as a protein that can be found in the vacuole (*falcipain-2*) was downloaded from the Protein Data Bank database (http://www.rcsb.org/) with a PDB ID of 3BPF. Molecular docking between bioactive compounds of the alkaloid group and drug control with the target protein was carried out using PyRx software (Autodock Vina). The results of molecular docking were combined with PyMOL DLP 3D and continued with visualization using AutoDock Tools and Discovery Studio Visualizer [25].

Statistical analysis

Data on the determination of total alkaloid content (in mg CE/g d.w. ethanolic extract) and antimalarial activity (in µg/mL) values are expressed as mean ± SD. One-way analysis of variance (ANOVA) followed by Tukey's HSD *post-hoc* test was used to determine the statistical significance of the comparison of sample activity with drug control on each Plasmodium strain using GraphPad Prism 10.1.0 software. A p -value of less than 0.05 was considered statistically significant. Data was collected with 3 repetitions.

Results and discussion

Screening and determination of alkaloid content

Identification of secondary metabolite compounds of the alkaloid group of the ethanolic extract of *C. amboinicus* leaves was carried out using commonly used standard reagents, namely Dragendorff, Mayer, and Wagner. The screening results with the Dragendorff reagent showed orange deposits, the Mayer reagent showed white deposits, and the Wegner reagent showed brown deposits in the test tube, which indicated positive content of alkaloid compounds. The presence of alkaloids in plant species is very limited and has distinctive characteristics. In the special extraction process, this group of compounds requires a special extraction technique [26]. The results of measuring the total content of alkaloids in the sample extract compared to caffeine as a standard alkaloid compound were 1.043 ± 0.001 mg CE/g d.w. ethanolic extract.

The alkaloid group in the metabolite pathway is a group of secondary metabolites that are naturally formed, characterized by the presence of nitrogen elements that characterize their presence. However, the presence of nitrogen atoms in natural compounds does not always mean they are alkaloid compounds; they can also be amino acids or other compounds and are referred to as pseudo alkaloid. The pharmacological effects of alkaloids are closely related to the arrangement of atoms in their chemical structure. Alkaloid groups with different chemical structures and functional entities will show diverse biological properties [27]. The process of alkaloid formation pathways in their biosynthesis pathways can be produced from the shikimate pathway, histidine and purine pathways, ornithine, lysine, and nicotinic acid pathways, and terpenoid and polyketide pathways. The presence of the content of this group of compounds is widely used in various treatments such as laxatives, antitussives, sedatives, dysentery, wounds, cuts, cholera, vaginal discharge, fractures, malaria, sprains, and various other diseases [28,29].

Alkaloid group compounds based on LC-HRMS

The secondary metabolite analysis approach using LC-HRMS/MS on untargeted ethanolic extract of *C. amboinicus* leaves samples. Analysis was carried out on all groups of compounds contained in the sample, including secondary metabolites of flavonoids, phenolics and polyphenols, steroids, tannins, terpenoids, and nitrogenated compounds (alkaloids and pseudo alkaloids). Currently, the method of analyzing secondary metabolite groups from natural materials using this analysis instrument has been widely applied [30]. The LC-HRMS instrument is a powerful and reliable tool in the analysis of compounds from natural materials, both food and plants, which allows identification and confirmation of the chemical structure of their constituents [31,32]. The LC chromatogram of the results of sample identification and analysis obtained 366 compound peaks, as shown in **Figure 1**.

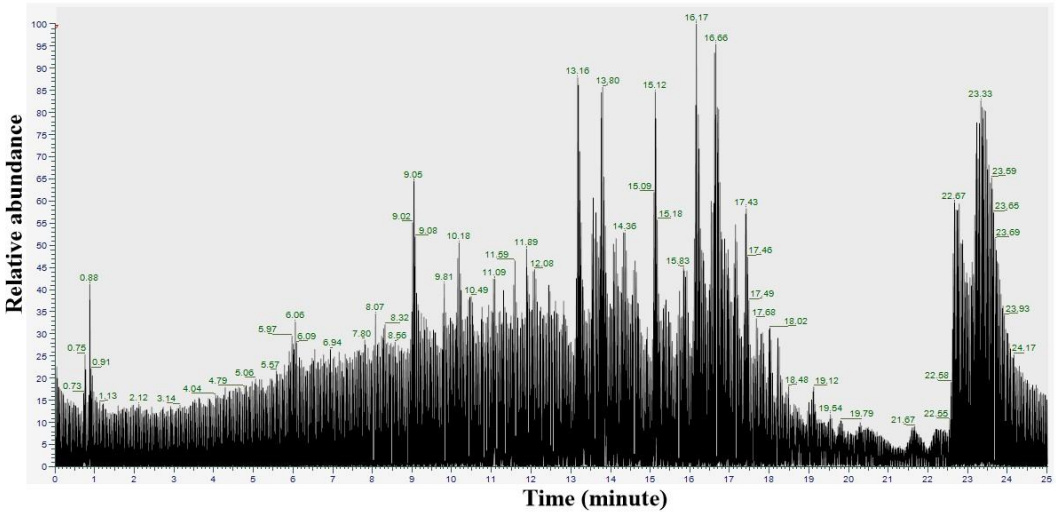

Figure 1 LC-HRMS chromatogram of the ethanolic extract of *C. amboinicus* leaves.

The ten compounds with the highest group area in the alkaloid group in sequence according to HRMS interpretation results are (1) choline, (2) pheophytin α, (3) 2,2,6,6-tetramethyl-1-piperidinol, (4) acridine-9(10*H*)-thione, (5) capsiamide, (6) 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), (7) 4-oleamidobutanoic acid, (8) cyclohexyl[4-(2-methoxyphenyl)piperidin-1-yl]methanone, (9) *N*-acetyl-L-phenylalanine, and (10) hydrocotarnine (**Table 1**). Each compound was identified using positive and negative ionization modes. Compounds identified in positive ionization include compounds (1), (2), (3), (5), (6), (7), (8) and (9); while compound (4) was identified using negative ionization mode. The ionization data generated by LC-HRMS comprehensively revealed the features of the detected compounds with mass measurements and chromatographic retention times, allowing for more accurate molecular formula determination, compound identification, and characterization [33]. This ionization mode data also aligns with the results of the experimental molecular weight (Exp) calculations from the instrument with existing reference data (Ref) of the compounds. The structure of compound N (alkaloids and pseudoalkaloids) in the ethanol extract of *C. amboinicus* leaves samples is shown in **Figure 2**.

Table 1 Bioactive compounds of the alkaloid group of the ethanolic extract of *C. amboinicus* leaves.

Comp	RT (min)	Compounds name	Formula	Group Area	Ionization mode	MW (g/mol)	
						Exp	Ref
1	0.747	Choline	$C_5H_{13}NO$	221470929.709	+	103.100	104.170
2	19.353	Pheophytin α	$C_{55}H_{74}N_4O_5$	146568612.403	+	870.565	871.200
3	8.944	2,2,6,6-Tetramethyl-1-piperidinol	$C_9H_{19}NO$	31091779.169	+	157.147	157.250
4	11.796	Acridine-9(10*H*)-thione	$C_{13}H_9NS$	25892758.223	–	211.045	211.280
5	14.750	Capsiamide	$C_{17}H_{35}NO$	17338936.829	+	269.272	269.500
6	10.129	1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)	$C_{12}H_{15}N$	21780193.451	+	173.121	173.250
7	15.112	4-Oleamidobutanoic acid	$C_{22}H_{41}NO_3$	15101228.486	+	367.308	367.600
8	6.912	Cyclohexyl[4-(2-methoxyphenyl)piperidin-1-yl]methanone	$C_{19}H_{27}NO_2$	13048168.021	+	301.204	301.400
9	9.148	*N*-Acetyl-L-phenylalanine	$C_{11}H_{13}NO_3$	6678939.520	+	207.090	207.230
10	10.122	Hydrocotarnine	$C_{12}H_{15}NO_3$	5604510.974	+	221.105	221.250

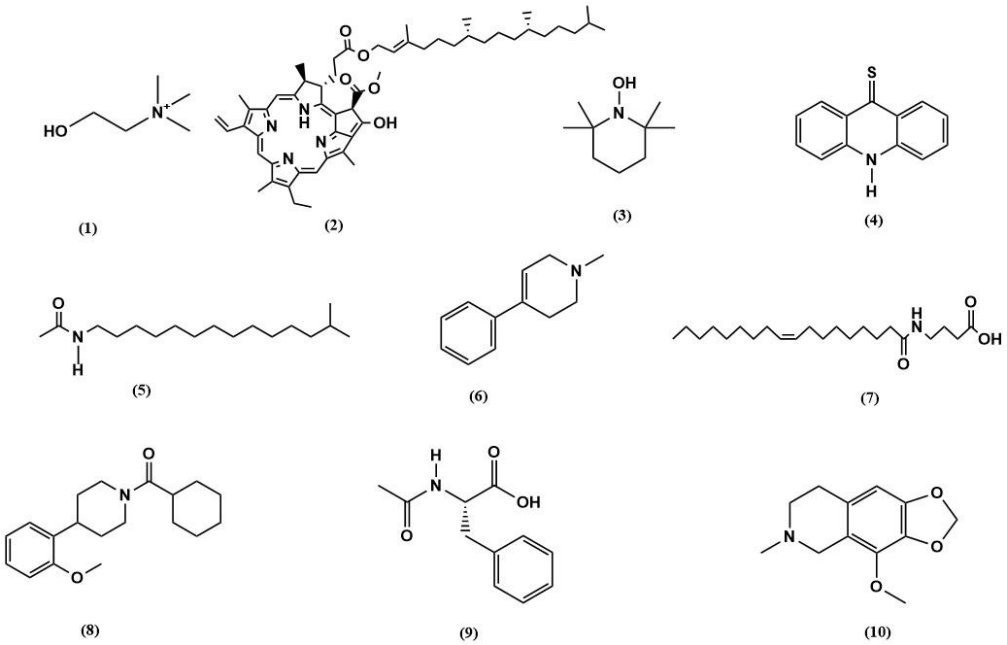

Figure 2 The structure of the main nitrogenated compounds identified in the ethanolic extract of *C. amboinicus* leaves.

The main nitrogenated compounds obtained from the analysis results using the LC-HRMS instrument were further identified, and various potential pharmacological activities were produced. Further identification of the nitrogenated compounds turned out to produce five alkaloid compounds, namely 2,2,6,6-tetramethyl-1-piperidinol (3), acridine-9(10*H*)-thione (4), 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (6), cyclohexyl[4-(2-methoxyphenyl)piperidin-1-yl]methanone (8), and hydrocotarnine (10), while pseudo-alkaloids include choline (1), pheophytin α (2), capsiamide (5), 4-oleamidobutanoic acid (7), and *N*-acetyl-L-phenylalanine (9). This nitrogenated compound provides a wide variety of pharmacological activities, including antioxidants, antimicrobial, antimalarial, antitumor, anticancer, antiproliferative, anti-inflammatory, and others. Identification of the various potential activities of each compound is presented in **Table 2**.

Table 2 Bioactive compounds of the alkaloid group of the ethanolic extract of *C. amboinicus* leaves.

No	Compounds name	Formula	Class of alkaloid	Potential activity	Reference
1.	Choline	$C_5H_{13}NO$	Pseudo alkaloids (nutrients)	Antioxidant, antimicrobial, neuroregenerative, neuroprotective, antimalarial	[34-36]
2.	Pheophytin α	$C_{55}H_{74}N_4O_5$	Pseudo alkaloids	Anti-inflammatory, antioxidant, anticancer, antimalarial	[37-39]
3	2,2,6,6-Tetramethyl-1-piperidinol	$C_9H_{19}NO$	Piperidine	Antimalarial, antiobesity, antioxidant	[40-42]
4	Acridine-9(10*H*)-thione	$C_{13}H_9NS$	Acridine derivative alkaloids	Antitumor, anticancer, antioxidant, antibacterial, antimalarial	[43-46]
5	Capsiamide	$C_{17}H_{35}NO$	Pseudo alkaloids (fatty acid compounds)	Antioxidant, antibacterial, antiviral, antiproliferative, antimalarial, antimutagenic, antiinflammatory, antidiabetic, antitumor, Alzheimer's	[47-51]
6	1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)	$C_{12}H_{15}N$	Pyridine	Antimalarial, anti-inflammatory, antibacterial, antiviral, antifungal, antileishmanial, anticancer, antioxidant	[52,53]
7	4-Oleamidobutanoic acid	$C_{22}H_{41}NO_3$	Pseudo alkaloids	Antioxidant, anticancer, anti-inflammatory	[54,55]

No	Compounds name	Formula	Class of alkaloid	Potential activity	Reference
8	Cyclohexyl[4-(2-methoxyphenyl)piperidin-1-yl]methanone	$C_{19}H_{27}NO_2$	Piperidine	Antibacterial, anti-inflammatory, anticancer, antimalarial	[56,57]
9	*N*-Acetyl-L-phenylalanine	$C_{11}H_{13}NO_3$	Pseudo alkaloids (amino acids)	Anti-inflammatory, disorders, malaria, bacterial infections, diuretic needs	[58-60]
10	Hydrocotarnine	$C_{12}H_{15}NO_3$	Benzylisoquinoline alkaloids	Anticancer, antioxidant, antimalarial	[61,63]

Antimalarial property against *P. falciparum* 3D7 and FCR3 strains

Antimalarial activity testing of the ethanol extract of *C. amboinicus* leaves was conducted *in vitro* using *P. falciparum* strain 3D7, which is sensitive to chloroquine but resistant to sulfadoxine, and FCR3 strain, which is sensitive to pyrimethamine and sulfadoxine but resistant to chloroquine and cycloguanil. Antimalarial activity testing was conducted in general to determine the growth inhibition potential of *P. falciparum*. The results of the test on the inhibition of *P. falciparum* strain 3D7 growth gave an activity value (IC_{50}) of 13.176 ± 0.006 µg/mL with a selectivity index (SI) value of 38.024 ± 0.005 and an IC_{50} against *P. falciparum* strain FCR3 of 49.683 ± 0.007 µg/mL with an SI value of 10.084 ± 0.001 (**Table 3** and **Figure 3**). Categories of antimalarial activity based on IC_{50} values include IC_{50} < 10 µg/mL (strong activity); IC_{50} 10 - 50 µg/mL (moderate activity); IC_{50} 50 - 100 µg/mL (weak activity); and IC_{50} > 100 µg/mL (inactive activity) [64]. Thus, the antimalarial activity of the ethanol extract of *C. amboinicus* leaves as a strong antimalarial category against *P. falciparum* strain 3D7 but moderate antimalarial activity against *P. falciparum* strain FCR3. The antimalarial activity value of the ethanol extract of *C. amboinicus* leaves is still weaker than chloroquine diphosphate as a common malaria drug, where the antimalarial activity value against *P. falciparum* 3D7 strain is 3.095 ± 0.010 µg/mL (SI 71.699 ± 0.200) and *P. falciparum* FCR3 strain is 3.199 ± 0.100 µg/mL (SI 69.299 ± 0.100). The ability of chloroquine diphosphate on both strains of *P. falciparum* is included in the strong category as an antimalarial, but inappropriate use of the drug can cause side effects and drug resistance. The ethanolic extract of *C. amboinicus* leaves has a selectivity index value of > 10 as an antimalarial, which indicates potential with good safety against normal cells so that it can be developed as an alternative antimalarial drug [65]. In addition, the resistance index caused by the use of the ethanolic extract of *C. amboinicus* leaves for malaria treatment based on data is lower (3.770 ± 0.001) compared to chloroquine diphosphate (10.327 ± 0.006) as a common drug in the treatment of malaria. Based on these data, it provides hope that the ethanolic extract of *C. amboinicus* leaves can be developed as a potential alternative malaria drug.

Table 3 Results of the ethanolic extract of *C. amboinicus* leaves antimalarial activity testing.

No.	Treatment	IC_{50} (µg/mL)		Resistance index	Selectivity index (SI)	
		P. falciparum 3D7 strain	*P. falciparum* FCR3 strain		CV-1/3D7	CV-1/FCR3
1.	Sample	13.179 ± 0.005	49.680 ± 0.006	3.770 ± 0.001	38.031 ± 0.010	10.089 ± 0.001
	Normal cells (CV-1)	501.217 ± 0.064	501.607 ± 0.575			
2.	Chloroquine diphosphate (CD/+)	3.095 ± 0.010	31.986 ± 0.997	10.327 ± 0.006	71.699 ± 0.200	69.299 ± 0.100
	Normal cells (CV-1)	221.692 ± 0.097	221.685 ± 0.008			

Note: activity values (IC_{50}) are expressed as means ± SD.

Orthodox drugs and herbal medicines as antiplasmodials have been reported to have mechanisms of action including inhibition of parasite nucleic acids and proteins, oxidative stress in parasites, inhibition of host glycolysis, inhibition of beta hematin (hemozoin) formation in hosts, and inhibition of *Plasmodium* species kinase enzymes. Beta hematin (hemozoin) is a by-product of hemoglobin degradation in malaria parasites by hemoglobinase containing toxic iron and causing oxidative stress in parasites. Reducing

hemozoin content through inhibition of its formation is likely to cause parasite death through oxidative stress by free radicals produced by reactive iron. Bioactive compounds in the ethanol extract of *C. amboinicus* leaves are likely to play a role in inhibiting membrane phospholipid biosynthesis in *P. falciparum* and interacting with metabolites produced from hemoglobin degradation in food vacuoles [40]. The results of the ethanolic extract of *C. amboinicus* leaves analysis using the LC-HRMS instrument, nitrogenated compounds such as choline compounds (1); pheophytin α (2); 2,2,6,6-tetramethyl-1-piperidinol (3); acridine-9(10*H*)-thione (4); capsiamide (5); 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (6); cyclohexyl[4-(2-methoxyphenyl)piperidin-1-yl]methanone (8); *N*-acetyl-L-phenylalanine (9); and hydrocotarnine (10) are reported to provide antimalarial activity.

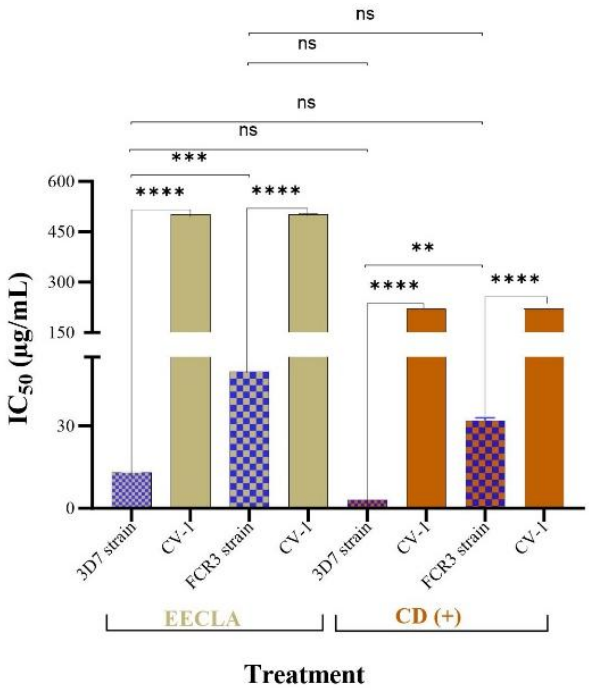

Figure 3 Analysis of sample activity test data against *P. falciparum* strain 3D7 and FCR3; activity test data are expressed as mean ± SD with significance levels of *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$.

ADMET and molecular docking investigation

ADMET screening and evaluation of potential bioactive compounds prior to their development as drug candidates is crucial. This step serves as a screening tool and provides an overview of the drug candidate's profile before proceeding to preclinical testing. The screening and evaluation utilize SwissADME and ProTox-3.0 web servers. All potential compounds developed as new drug candidates must at least meet Lipinski's five rules of suitability for oral administration. Compliance with these rules will ensure effective efficacy, distribution, metabolism, optimal activity, specification, and selectivity of oral drugs. ADMET information of potential bioactive compounds is shown in **Table 4**.

Table 4 ADMET prediction of bioactive compounds from the ethanolic extract of *C. amboinicus* leaves.

Parameters	Bioactive compounds								
	1	**2**	**3**	**4**	**5**	**6**	**8**	**9**	**10**
Lipinski's rules									
MW (g/mol)	104.17	871.20	157.25	211.28	269.47	173.25	301.42	207.23	221.25
HBD	1	2	1	1	1	0	0	2	0
HBA	1	8	2	0	1	1	2	3	4
LogP	–1.86	9.91	1.84	3.59	5.04	2.50	3.61	1.09	1.73
TPSA (Å^2)	20.23	121.84	23.47	47.88	29.10	3.24	29.54	66.40	30.93

Parameters	Bioactive compounds								
	1	**2**	**3**	**4**	**5**	**6**	**8**	**9**	**10**
Pharmacokinetic Data									
GI absorption	Low	Low	High	High	High	Low	High	High	High
BBB permeant	No	No	Yes	Yes	Yes	Yes	Yes	No	Yes
P-gp substrate	No	No	No	No	No	No	No	No	Yes
CYP1A2 inhibitor	No	No	No	Yes	Yes	No	No	No	Yes
CYP2C19 inhibitor	No	No	No	Yes	No	No	No	No	No
CYP2C9 inhibitor	No	No	No	No	No	No	No	No	No
CYP2D6 inhibitor	No	No	No	No	No	Yes	Yes	No	Yes
CYP3A4 inhibitor	No	No	No	No	No	No	No	No	No
Skin Permeation (cm/s)	–7.22	–3.29	–6.29	–5.05	–3.36	–5.43	–5.29	–6.90	–6.53
Bioavailability score	0.55	0.17	0.55	0.55	0.55	0.55	0.55	0.85	0.55
Toxicity Prediction									
LD_{50} (mg/kg)	1,391	40	1,394	360	3,200	150	2,000	1,000	750
Class	4	2	3	4	5	3	4	4	4

Based on Lipinski's rule, the bioactive compounds acridine-9(10H)-thione (4) and N-acetyl-L-phenylalanine (9) meet the requirements, while compounds (1), (3), (6), (8), and (10) do not meet the TPSA requirements in the range of 40 - 130 Å^2; bioactive compound (5) does not meet the YPA range and logP > 5; and bioactive compound (2) does not meet the logP value, and the molecular weight is greater than 500 g/mol. Predicted gastrointestinal (GI) absorption is high except for bioactive compounds (1) and (2), which ensure effective systemic exposure; predicted high BBB permeability and not being identified as P-glycoprotein (P-gp) substrates for compounds (3), (4), (5), (6), (8), and (10) indicate reduced efflux potential and better bioavailability. Furthermore, pharmacokinetic predictions show inhibition of key cytochrome P450 enzymes (CYP1A2, CYP2D6, CYP2C19, CYP2C9, and CYP3A4), indicating metabolic stability and providing information on its pharmacokinetic significance. Toxicity predictions (LD50) provide information on the estimated dose of bioactive compounds that cause death in 50% of test animals in a population. The smaller the LD50 value, the higher the level of toxicity. Bioactive compound (2) has the lowest value, indicating the highest toxicity, and is included in class 2 category. Based on the description of the ADMET predictions carried out, the compounds acridine-9(10H)-thione (4) and N-acetyl-L-phenylalanine (9) provide more potential opportunities to develop as malaria drug candidates.

A molecular docking study was conducted on malaria target protein using the AutoDock Vina PyRx program to predict the various possible interactions and binding modes of the bioactive compounds from the ethanolic extract of *C. amboinicus* leaves to the receptor by comparing chloroquine as a common drug for malaria and native ligand (CID 5288145). The code and protein as a receptor were obtained from the PDB database (www.rcsb.org), PDB ID 3BPF for *falcipain-2*, a protein that can be found in *P. falciparum* food vacuoles involved in hemoglobin hydrolysis [66,67] *P. falciparum* is a parasite responsible for malaria and the deadliest; it replicates in its host erythrocytes, causing extensive DNA damage, so it must be repaired efficiently to ensure parasite survival [68]. The results of molecular docking of N, native, and chloroquine (control drug) compounds to the *falcipain-2* receptor are shown in **Table 5**, and the complex structure and types of interactions that occur are shown in **Figure 4**.

Table 5 Optimization and interaction studies of molecules with the *falcipain-2* receptor using computational approaches.

Compounds	Structure optimization		Molecular docking result		Interactions		
	Energy (kcal/mol)	Dipole moment (Debye)	Affinity (kcal/mol)	RMSD (Å)	H-Bond	van der Waals-Bond	Other-Bond
(1)	–327.05	1.01	–3.7	0.76	His174	Gly83, His174	Asp234

Compounds	Structure optimization		Molecular docking result		Interactions		
	Energy (kcal/mol)	Dipole moment (Debye)	Affinity (kcal/mol)	RMSD (Å)	H-Bond	van der Waals-Bond	Other-Bond
(2)	–2,718.95	7.43	–6.4	1.18	Asn173	His174	Cys42, Trp206, His174
(3)	–481.70	2.54	–5.1	1.98	Cys42	-	Leu84, Ile85, Leu172, Ala175
(4)	–948.76	6.50	–6.5	0.55	-	-	Cys42, Ile85, Leu84, Ala175, Asp234
(5)	–794.58	3.50	–4.7	1.09	Thr64	-	Lys34, Pro105, Tyr106
(6)	–518.19	0.87	–5.8	1.12	-	-	Lys34, Tyr106
(7)	–1,112.96	2.59	–5.0	1.65	Thr64, Asp101	-	Lys34, Phe45, Pro105, Tyr106, Gly102
(8)	–940.36	5.22	–6.5	1.43	Asp35	-	Pro32, Lys34, Tyr106
(9)	–703.56	3.71	–6.0	0.68	His19, Asn134, Lys137, Glu138	-	Arg12
(10)	–742.59	1.87	–5.9	1.08	Cys80, Asn112	Ser108, Ala110	Val71, Cys80
Chloroquine	–1,319.10	7.34	–5.8	0.92	Val71, Ser108	Cys80	Val71, Phe75, Cys80
Native ligand	–1,232.71	3.23	–6.0	1.43	Val71, Asp72, Gly79, Cys80, Asn81, Ser108, Asn112	-	Asp72, Gln68

This molecular docking study research first conducted a computational investigation to optimize the shape of the chemical geometric structure of each compound by applying the Hartree-Fock (HF) theory function, DFT method, and 3-21G basis set using GaussView 5.0 [69]. The optimization of the structure shape aims to obtain a 3D structure with minimal energy, thermal stability, and molecular charge and biochemical behavior of each compound based on quantum mechanical calculations, so that it is expected to be in the same structural condition in the body [25]. The optimization results presented show all compounds produce negative optimization energy, which indicates thermodynamic stability. The highest stability among nitrogenated compounds is compound (2), with a stability energy of –2,718.95 kcal/mol and a dipole moment of 7.43 D, while chloroquine has –1,319.10 kcal/mol and a dipole moment of 7.34 D, and the native ligand has –1,232.71 kcal/mol and a dipole moment of 3.23 D.

After optimization of each N compound of the ethanolic extract of *C. amboinicus* leaves, chloroquine, and native ligand were docked for 50 LGA in a grid box with dimensions of 5×5×5 nm^3 to find the most stable conformation and interaction with amino acid residues in the active site of the *falcipain-2* receptor (PDB ID 3BPF). The results of molecular docking and RMSD values, from all nitrogenated compounds, chloroquine, and native ligands showed values less than 2.00 Å, indicating the validity of the molecular docking protocol [69]. The most stable conformation and best binding mode of each compound with the *falcipain-2* receptor are shown in **Table 5** and **Figure 4**. The binding energy of ten nitrogenated compounds with higher negative values than chloroquine (–5.8 kcal/mol, RMSD 0.92 Å) are compounds (4), (8), (2), (9), and (10) with binding energy values of –6.5, –6.5, –6.4, –6.0 and –5.9 kcal/mol, respectively. As shown in **Figure 4**, compound (4) does not have hydrogen and van der Waals bonds, but the complex structure is stabilized by pi-sigma, pi-sulfur, pi-alkyl, pi-anion, and unfavorable donor-donor interactions. Compound (8) has a hydrogen bond with Asp35 and is stabilized more by pi-pi stacked, pi-alkyl, and alkyl interactions. On the other hand, compound (2) generates hydrogen bonds with Asn172, van der Waals bonds with His174, and other hydrophobic interactions including alkyl, pi-alkyl, and pi-pi T-shaped interactions. Compound (9) forms four hydrogen bonds with His19, Asn134, Lys137, and Glu138; and several pi-anion and pi-cation interactions. Meanwhile, compound (10) shows two hydrogen bonds with Cys80, andAsn112, two van der Waals bonds with Ser108 and Ala110, and some alkyl and pi-alkyl interactions. In contrast, chloroquine has two hydrogen

bonds with Ser108 and Val71 and a van der Waals bond with Cys80 while the native ligand has seven hydrogen bonds with Val71, Asp72, Gly79, Cys80, Asn81, Ser108, and Asn112, which is supported by attractive charge and unfavorable donor-donor interactions. The molecular docking data reveal that each compound showed unique non-covalent interactions; thus, yielding different in silico binding energies and *in vitro* IC_{50} values. Different binding modes of the extracted compounds may explain why the ethanolic extract exhibited lower resistance index than chloroquine, which was remarkable.

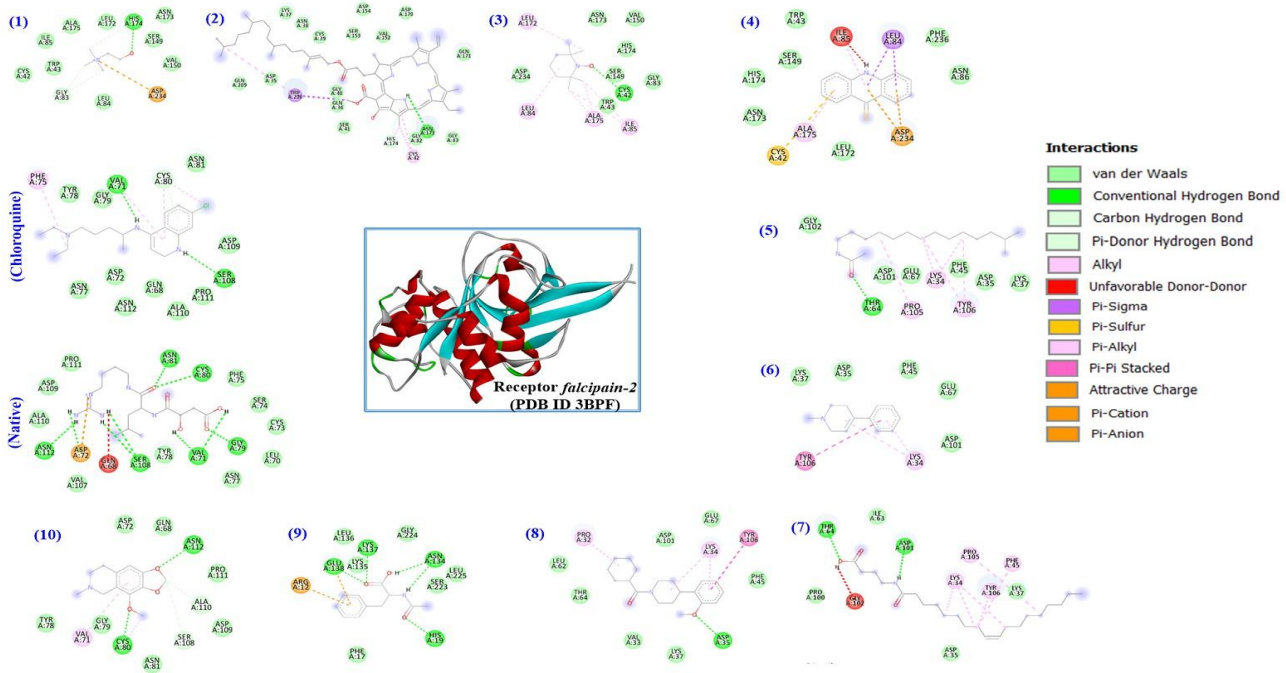

Figure 4 Visualization of the interaction of nitrogenated compounds of the ethanolic extract of *C. amboinicus* leaves in the active site of the *falcipain-2* receptor.

Conclusions

In this study, ten nitrogenated compounds were found that are included in the alkaloid group of the ethanolic extract of *C. amboinicus* leaves, which have the potential to provide antimalarial activity and provide inhibition of malaria *falcipain-2* receptor. These compounds seem interesting to be further developed for the development of new antimalarial agents as alternative substituents or complement to the availability of existing malaria drugs. These findings provide supporting evidence for the traditional use of *C. amboinicus* leaves as a malaria drug. The structural model and interaction of each compound that occurs with the *falcipain-2* receptor provide computational information for the design of future antimalarial drugs.

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Declaration of Generative AI in Scientific Writing

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CRediT Author Statement

Kasta Gurning: Conceptualization, Methodology, Validation, Funding acquisition, and Writing –original draft. **Yehezkiel Steven Kurniawan**: Data curation, Formal analysis, Investigation, Validation, Visualization, and Writing –original draft. **Friska Septiani Silitonga**: Data curation, Formal analysis, Investigation, Validation, and Visualization. **Suratno**: Data curation, Formal analysis, and Visualization. **Gian Primahana**: Data curation, Formal analysis, Investigation, Validation, and Visualization. **Charlie Ester de Fretes**: Data curation, Formal analysis, Investigation, Validation, and Visualization. **Mario Rowan Sohilait**: Data curation, Formal analysis, Investigation, Validation, and Visualization. **Endang Astuti**: Data curation, Formal analysis, Investigation, Validation, Visualization, and Writing –review & editing. **Winarto Haryadi**: Conceptualization, Resources, Software, Funding acquisition, and Writing –review & editing.

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