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INHIBITORY ACTIVITY OF BIOACTIVE COMPOUNDS IN BLACK SEA CUCUMBER (*HOLOTHURIA ATRA*) AGAINST *FALCIPAIN-2* PROTEIN IN *PLASMODIUM FALCIPARUM* AS ANTIMALARIA BASED ON *IN SILICO* STUDY

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ABSTRACT

Background: Various marine biota in Indonesia can be used as alternatives for malaria treatment, one of which is *Holothuria atra*. Indonesia is reported as the second-largest contributor to malaria in the Asian. *Plasmodium falciparum* is the most common malaria-causing parasite in Indonesia. The high incidence of resistance of the malaria parasite *Plasmodium falciparum* to malaria drugs makes the bioactive compounds in *Holothuria atra* a very advantageous discovery as a new antimalarial drug. **Objective:** to determine the inhibitory effects of active compounds in *Holothuria atra* on the development of *Plasmodium falciparum* based on in silico studies. **Method:** The research design used was a one-shot experimental study, contained several stages, including protein and ligand preparation; prediction of bioactive compound potential and target protein; molecular docking and visualization of docking results; and prediction of drug-likeness and absorption, distribution, metabolism, excretion, toxicity (ADMET) **Results:** Based on the test results, the active compounds are chlorogenic acid, catechin, rutin, coumaric acid, pyrogallol, and ascorbic acid, show inhibition of *Falcipain-2*, which is used to degrade hemoglobin and erythrocytes in the acidic digestive vacuole. Chlorogenic acid and catechin have the highest binding affinity values. The ADME analysis show that four active compounds, are catechin, coumaric acid, pyrogallol, and ascorbic acid, comply the Lipinski criteria, making them potential candidates for oral drugs. Catechin is the safest compound classified as class 6 toxicity (non-toxic). **Conclusion:** The research that has been carried out describes the results, that *Holothuria atra* has the potential to be an antimalarial drug.

INTRODUCTION

Malaria is a disease caused by the *Plasmodium* species parasite through the bite of infected female Anopheles mosquitoes. This disease is a common cause of fever in travelers from endemic countries [1]. According to WHO reports, in 2022, there were 249 million malaria cases in 85 endemic countries, showing an increase from 2021. Most cases, 233 million (94%), were reported in Africa. And the mortality is estimated at 608,000 cases, with over 50% occurring in four countries. From 2000 to 2022, Southeast Asia saw a 77% decrease in malaria cases, but there was an increase noted in several countries, including Indonesia [2]. Indonesia reported the second-highest number of malaria cases in Asia, with approximately 790,598 cases estimated in 2022, especially showed a rise in cases, with Papua accounting for 89% of the total. Investigations revealed the common parasites causing malaria in Indonesia were *Plasmodium falciparum* [3]. Malaria infections from *Plasmodium falciparum* can cause dangerous manifestation, such as severe anemia, malaria cerebral, and death [4].

The therapeutic treatment for malaria follows Indonesia's national guidelines, primarily using Artemisinin-Based Combination Therapy (ACT) because of resistance to chloroquine and sulfadoxine-pyrimethamine has been identified in Indonesia [5]. ACT is globally recognized as the first-line treatment for uncomplicated falciparum malaria, even new

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compounds will not be available soon. This is proven by the resistance of *Plasmodium falciparum* to artemisinin (ART-R). It was found over a decade ago in the Greater Mekong Subregion, affecting ACT partner drugs. The mechanism shows resistant parasites surviving artemisinin attacks, with mutations in the *P. falciparum* Kelch13 gene. Differences in genomic, transcriptomic, and proteomic profiles indicate distinct cellular responses to artemisinin exposure, leading to decreased hemoglobin metabolism and reduced effectiveness of artemisinin [6].

Recent findings on the resistance to first-line malaria drugs highlight the need for effective alternative treatments, one of them is *Holothuria atra* as biomarine for alternative therapies. The active compounds in the extract of *Holothuria atra* include chlorogenic acid, pyrogallol, routine, coumaric acid, catechin, and ascorbic acid[7] .The body wall is the main source of chlorogenic acid, containing over 93% by weight [8].

Plasmodium falciparum uses falcipain, a cysteine protease that plays a role in the parasite's life cycle to obtain nutrients and stability by degrading red blood cell proteins, especially host hemoglobin in acidic vacuoles. In addition, the fact that the protease being studied comes from a strain of *Plasmodium falciparum* that is resistant to ACT [9]. So, the inhibitory activity of FP-2 to prevent parasite maturation indicates that this protein can be a valuable target for the design of new antimalarial drugs [10]. This study will explore innovative pharmacological molecules using in silico methods to analyze interactions with FP-2 for effective antimalarial drug development [11]. The objective of the research was to prove the inhibitory effect of bioactive compounds in *Holothuria atra* on the *Falcipain-2* protein in *Plasmodium falciparum*.

METHODS

This research was using the one-shot experimental study type with in silico as the method and was done in Laboratorium Biomolekuler & Bioinformatika INBIO Situbondo, East Java, Indonesia from August until October 2023. The independent variable was the active substances in *Holothuria atra*, the dependent variable was the development of *P. falciparum*.

Protein structure and ligand

The first step involves data mining bioactive compounds from *Holothuria atra* using the PubChem database (https://pubchem.ncbi.nlm.nih.gov/) to obtain the SMILES of each compound [12]. The 3D crystal structure of the target protein, *Falcipain-2* (PDB ID: 3BPF), is retrieved from the Protein Data Bank (PDB) (https://www.rcsb.org/) [13].

Potential prediction of the substances

Active compounds from Holothuria atra will be analyzed for their antimalarial potential using the WAY2DRUG PASSOnline Prediction web server (http://www.pharmaexpert.ru/passonline/predict.php) [14]. The quantitative structure-activity relationship (QSAR) approach compares input structures with database structures, leading to a Probability To Be Active (Pa) value indicating the likelihood of pharmacological activity [15]. Compounds with Pa values >0.3 will undergo molecular docking analysis to assess their potential as antimalarial agents [14].

Moleculer docking

Predictions about weak and strong affinities are made through docking analysis, which shows receptor-ligand interactions and affinity energy to assess the potential of compounds as antimalarials [14]. A more negative value indicates a stronger relationship [12]. After obtaining the target protein structure and bioactive compounds, the protein is prepared by removing water molecules in Discovery Studio 2019, while ligands undergo energy minimization in PyRx v. 0. 9. 8 [16]. The docking process uses Autodock Vina integrated with PyRx v. 0. 9. 8 [17]. If the tested bioactive compound value approaches the control value, it shows similar activity as a protein target inhibitor [18]. Docking focuses on the *Plasmodium falciparum Falcipain-2* (FP-2) protein target with the inhibitor control compound E64, and interactions are visualized using BioVia Discovery Studio 2019 [16].

Prediction of absorption, distribution, metabolism, and excretion (ADME)

ADME prediction for each ligand is based on Lipinski's rule of 5, analyzed using SwissADME (http://www.swissadme.ch/) [19]. This rule assesses drug-likeness and oral activity of bioactive compounds. Good membrane permeability is

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evaluated through that rules : Less than 5 Log P; less than equal to 5 hydrogen bond donors (HBD); less than equal to 10 hydrogen bond acceptors (HBA); molecular weight is less than 500 g mol^{-1} and less than 140 $Å^2$ Topological Polar Surface Area (TPSA) [20]. Additional molecular descriptors include human intestinal absorption and blood-brain barrier permeability [21]. Higher HIA indicates greater absorption.

Prediction of toxicity

The drug-likeness character showing inhibitor effects on parasite cultures is analyzed for toxicity using ProTox-II (https://tox-new.charite.de/protox_II/index.php?site=compound_input) [22]. Toxicity prediction is evaluated using the LD50 measure, indicating lethal doses, where 50% of the tested animal population dies due to exposure to the tested compound. ProTox-II categorizes toxicity into six classes based on LD50 values, ranging from highly toxic to non-toxic when ingested. Based on the Globally Harmonized System (GHS), there are six levels of toxicity in ProTox-II based on the LD50 value classified as follows:

a. Class 1 : fatal if swallowed ($LD_{50} \leq 5$)
b. Class 2 : fatal if swallowed ($5 < LD_{50} \leq 50$)
c. Class 3 : toxic if ingested ($50 < LD_{50} \leq 300$)
d. Class 4 : dangerous if swallowed ($300 < LD_{50} \leq 2000$)
e. Class 5 : may be harmful if swallowed ($2000 < LD_{50} \leq 5000$) 6)
f. Class 6 : non-toxic if swallowed ($LD_{50} > 5000$)

The higher the level of toxicity of a compound, the lower the toxicity when ingested [23].

RESULTS

Active substances in *Holothuria atra* and its potential prediction

Table 1 shows that all active compounds in *Holothuria atra* extract include chlorogenic acid, pyrogallol, routine, coumaric acid, catechin, and ascorbic acid[7] have antiparasitic activity. The Probability To Be Active (Pa) indicates catechin has a low potential as an antimalarial agent, while coumaric acid has the highest Pa value of 0,446.

Table 1. Analysis of the Bioactive Compound Potential of *Holothuria atra*

Active compounds	ID	Antiparasitic (Pa)
Chlorogenic Acid	1794427	0.41
Pyrogallol	1057	0.329
Rutin	5280805	0.393
Coumaric Acid	637542	0.446
Catechin	9064	0.191
Ascorbic Acid	54670067	0.306

Prediction of active substances pathway against FP-2

E64, a control ligan, was used in a study to inhibit FP-2, a protein that affects cellular activity, merozoit movement, membrane damage, and merozoit aggregation[24]. The results in the table 2 above shows that the docking results of chlorogenic acid and catechin compounds have the strongest binding affinity (-6.3) against the target protein *Plasmodium falciparum Falcipain-2* (FP-2).

Table 2. Binding Affinity between Protein Marker with Ligand of *Holothuria atra* and Control

Active compounds	*binding affinity* (Kcal/mol)
E64	-5,5
Chlorogenic Acid	-6,3
Catechin	-6,3
Rutin	-5,9
Ascorbic Acid	-5,1
Pyrogallol	-4,8
Coumaric Acid	-4,4

Table 3 shows that chlorogenic acid, ascorbic acid, and pyrogallol are the most stable compounds because they form the most strong hydrogen bonds and have many similarities between the amino acid residues of the test ligand and the control ligand, indicating a higher level of activity similarity[25].

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Table 3. Interaction of Amino Acid Residues between Marker Protein with Ligand of *Holothuria atra* and Control

Active compounds	Interaction				
	Van der Waals	Conventional Hydrogen Bond	Carbon Hydrogen Bond	Hydrophobic	Unfavorable
E64	GLY82 ASN81 TYR78 SER149 LEU84 ILE85 ALA175 HIS174 ASN173 SER41 GLN36	CYS42 GLY83	TRP43 GLY83 GLY40 LEU172		
Chlorogenic Acid	**ILE85 SER149** ILE148 LEU172 **SER41 GLY82** TRP43 CYS80 GLY83 **LEU84**	GLN36 ASN81 **CYS42** GLY40		ASN173 HIS174 ALA175	
Catechin	GLY40 **ASN81** **GLY82** GLY83 VAL150 **LEU84** LEU172 ASP234 **ILE85** TRP43 **GLN36**		SER149		
Rutin	LEU172 VAL150 **HIS174 ALA175** **ASN173 ILE85** TRP43 CYS42 GLY83 GLY40 **GLY82 LEU84** **ASN81**		SER149		
Ascorbic Acid	ASP234 ILE148 **SER149 ALA175** LEU172 VAL150 **ASN173** CYS42 **GLY82** TRP43	**GLY83** ILE85 HIS174	LEU84		GLY83
Pyrogallol	TRP43 **ASN173** LEU172 VAL150 **SER149 ILE85** **LEU84**	**CYS42 GLY83** HIS174		CYS42 ALA175	
Coumaric Acid	**TYR78 ASN173** LEU172 ASP234 **SER149** VAL150 **ALA175** TRP43 **HIS174** CYS42 **ASN81 GLY82**	**GLY83**			

Information : **bold** datas above indicate the same amino acid residue from control and ligand

Prediction of Absorption, Distribution, Metabolism, and Excretion (ADME)

According to the results below (table 4), there are bioactive compound that does not fulfill the criteria of Lipinski's rule, namely chlorogenic acid and rutin, so that compounds are not suitable to be used as medicine, whereas the compound with the highest potential to be developed into a drug is coumaric acid because it fulfills the Lipinski criteria.

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Table 4. ADME Analysis of Active Compounds in *Holothuria atra* (chlorogenic acid, pyrogallol, routine, coumaric acid, catechin, and ascorbic acid)

Lipinski's rule	Active compounds					
	Chlorogenic Acid	**Pyrogallol**	**Rutin**	**Coumaric Acid**	**Catechin**	**Ascorbic Acid**
Molecular weight	354,31	126,11	610,52	164,16	290,27	176,12
Hydrogen bond acceptors	9	3	16	3	6	6
Hydrogen bond donors	6	3	10	2	5	4
TPSA	164,75	60,69	269,43	57,53	110,38	107,22
WLOGP	-0,75	0,8	-1,69	1,38	1,22	-1,41
GI Absorption	Low	High	Low	High	High	High
BBB permeant	-	+	-	+	-	-
P-gp substrate	-	-	+	-	+	-
Bioavaibility	0,11	0,55	0,17	0,85	0,55	0,56

Lipinski's rule of :
1. Less than 5 Log P
2. Molecular weight is less than 500 g mol^{-1}
3. Less than equal to 5 hydrogen bond donors
4. Less than equal to 10 hydrogen bond acceptors
5. less than 140 $Å^2$ Topological Polar Surface Area (TPSA)

Prediction of toxicity

Based on the table 5, the toxicity test results indicate that the safest bioactive compound in *Holothuria atra* is catechin with an LD50 of 10,000 $mg.kg^{-1}$ wt, belonging to toxicity class 6, while the most dangerous bioactive compound is pyrogallol, classified as toxicity class 3.

Table 5. Toxicity Prediction based on LD50 and Toxicity Classification

Toxicity parameter	Active compounds					
	Chlorogenic Acid	**Catechin**	**Rutin**	**Ascorbic Acid**	**Pyrogallol**	**Coumaric Acid**
Prediction of LD_{50} ($mg.kg^{-1}$ wt)	1190	10000	5000	3367	300	2850
Prediction of toxicity (class)	4	6	5	5	3	5

DISCUSSION

Active substances in *Holothuria atra* and its potential prediction

The active compounds contained in the extract of the species Holothuria atra, including chlorogenic acid, pyrogallol, rutin, catechin, coumaric acid, and ascorbic acid, have antiparasitic activity. Based on the antiparasitic value (Pa), there are two compounds with strong potential as antimalarials, namely chlorogenic acid and coumaric acid, with the highest Pa values > 0.3. However, there is one bioactive compound (catechin) with a Pa value < 0.3, indicating that the compound is less potent as an antimalarial agent (Table 1).

Chlorogenic acid has not been studied for its potential as an antimalarial in silico, but it has shown promise as an antiplasmodial in vivo and in vitro. Caffeic acid and chlorogenic acid, along with seven of their synthetic derivatives, demonstrated in vitro effectiveness against *P. falciparum*. The study used

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artemisinin as a positive control, showing an IC50 of 3. 9 ng/ml. In vivo study exhibit that ethyl caffeate inhibits *P. berghei* growth and works similarly to artemisinin [26]. Research using another in silico study explains that chlorogenic acid has an antiparasitic value (Pa) of 0.410 (>0.3)[18]. Pyrogallol is produced from the hydrolysis of tannin from gallic acid and can inhibit the enzyme *Dihydrofolate Reductase* (DHFR) in *P. falciparum*, which helps stop parasite growth[27]. It generates free radicals and reactive oxygen species (ROS), which kill the parasite by weakening its antioxidant defenses[21]. Pyrogallol can also alter the pH of the digestive vacuole in *P. falciparum* by inhibiting the proton pump[28]. Another study results show pyrogallol has an antiparasitic value of 0.329, lower than artemisinin's value of 0. 857, indicating its effectiveness against malaria parasites[18]. The bioactive compound rutin can inhibit *Falcipain-2*, a protease used by trophozoites to degrade hemoglobin. In silico studies show that rutin from Artocarpus lakoocha has the best docking score[19].

Coumaric acid is a natural phenolic compound known for its pharmacological activities as an antioxidant, antimicrobial, anti-inflammatory, antineoplastic, antimalarial, and neuroprotective agent. Its derivative, methyl p-coumarate, shows activity against *P. falciparum* by inhibiting its folate synthesis. Based on in vivo study, the effectiveness is measured by their effective concentration (EC50)[29]. Additionally, in vitro study exhibits methyl-4-hydroxycinnamate (M4H) can inhibit resistant *P. falciparum* strain development and reduce parasitemia levels[30]. Coumaric acid has an antiparasitic value of 0. 446, the highest Pa value same with other in silico study[18]. Catechin is a polyphenol found in various medicinal plants like Osyris alba and Camellia sinensis. It has pharmacological activities, including antioxidant and anti-inflammatory effects[31]. Catechin can inhibit malaria-causing parasites by blocking specific enzymes such as *P. falciparum Orotidine 5-Monophosphate Decarboxylase* (PfOMPDC), which facilitates pyrimidine synthesis in the life cycle of the parasite, but showing a lower antiparasitic value than artemisinin[18]. Studies reveal catechin can suppress malaria growth and affect different parasite stages effectively[27]. Ascorbic acid is known as vitamin C and an antioxidant[32]. It has potential as an antimalarial agent by inhibiting the enzyme PfOMPDC indirectly through CO2. Increased CO2 speeds up ascorbic acid formation, leading to competition for CO2. Studies show ascorbic acid has an antiparasitic value of 0. 306, lower than artemisinin's value of 0. 857[18].

Prediction of active substances pathway against FP-2

The analysis of the predicted mechanism of action of the bioactive compound *Holothuria atra* against Falcipain-2 can be observed through molecular docking tests in the form of binding affinity and amino acid residues resulting from the interaction between the receptor and the test ligand. E64 is a control compound has been shown to be an inhibitor of FP-2. It is a permanent cysteine protease inhibitor that blocks the FP-2 food vacuole, merozoite release, red blood cell membrane damage, destroys merozoites by damaging their membranes, and increases merozoite vulnerability[24].

Based on table 2, the top three bioactive compounds in the molecular docking results are chlorogenic acid, catechin, and rutin, with binding affinity values lower than the control E64. The inhibition potential of these compounds against FP-2 is better than the control (-5.5). Other research by Moelyadi have also proven the same[18].

After obtaining the structure of the target protein and bioactive compounds, protein is prepared by removing water molecules using Discovery Studio 2019, resulting in amino acid residues that form the active site for receptor-ligand binding, so that can inhibit receptor[16]. All control and test ligands can interact with FP-2 at its active site form some interactions, one of them is conventional hydrogen bonds, that crucial for forming stable ligand-protein interactions. The more strong hydrogen bonds formed, the greater the ligand's affinity for the receptor[33]. Table 3 shows that chlorogenic acid, ascorbic acid, and pyrogallol are the most stable compounds because they form the most strong hydrogen bonds. Chlorogenic acid forms four bonds, ascorbic acid and pyrogallol form three bonds, while coumaric acid forms only one strong hydrogen bond. Each compound has one until two (pyrogallol) amino acid residues similar to the control. Therefore, that compounds have a high level of activity similarity.

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Prediction of Absorption, Distribution, Metabolism, and Excretion (ADME)

The oral bioavailability of a compound with good membrane permeability is seen from the Log P value, a parameter used to evaluate the lipophilicity or hydrophobicity of a compound, the higher the Log P, the more lipophilic or hydrophobic the compound is, allowing it to penetrate the lipid bilayer cell membrane and making it less soluble in water[34]; topological polar surface area (TPSA), for evaluating the polarity of a compound, the higher the TPSA value (hydrophilic), the less effective it is at penetrating the cell membrane and reducing bioavailability[35]; molecular weight (MW) shows the compounds with high molecular weight will inhibit gastrointestinal absorption and cell penetration[34]; hydrogen bond acceptor (HBA) and hydrogen bond donors (HBD), the more hydrogen bonds, the more energy is required for the compound to be absorbed, thus reducing the compound's ability to penetrate the cell membrane[36].

To complete the evaluation of in silico results, other molecular descriptors are needed, such as the presence or absence of blood-brain barrier (BBB) permeability, high or low gastrointestinal (GI) absorption, bioavailability score[21], and P-glycoprotein (P-gp), which is a protein located in the cell membrane that removes foreign substances from cells, reducing drug absorption and bioavailability[37].

Among all the compounds of *Holothuria atra*, 5 of them fulfill the Lipinski criteria, are pyrogallol, rutin, coumaric acid, catechin, and ascorbic acid, while chlorogenic acid and rutin do not fulfill these criteria, making these compounds less suitable for oral medication. Pyrogallol and coumaric acid are compounds with high GI absorption, high blood-brain barrier (BBB) permeability, not P-gp substrates, and high bioavailability. Because these compounds have the ability to cross the blood-brain barrier (BBB), they can be used as treatments for cerebral malaria. Catechin and Ascorbic acid are compounds with high GI absorption and high bioavailability but are difficult to penetrate the blood-brain barrier (BBB) and are P-gp substrates. The results of the above study are similar to previous research that explains that the six active components from *Holothuria atra*, or other species like *Phoenix dactylifera*[38], *Artocarpus lakoocha*[19], *T. diversifolia*, *B. sapida*, *I. gabonensis*[39], *and Murraya koenigii*[39] exhibit the same Lipinski criteria and molecular descriptors.

Prediction of toxicity

Artemisinin, the standard malaria drug, has a toxicity of 900 $mg.kg^{-1}$wt in the class 4 toxicity category (dangerous if swallowed)[40]. Compounds such as chlorogenic acid, catechin, rutin, coumaric acid, and ascorbic acid have higher LD_{50} values compared to artemisinin, while pyrogallol has a lower LD_{50} value. Catechin is considered the safest with an LD_{50} of 10,000 $mg.kg^{-1}$wt in the toxicity class 6 (non-toxic) category, followed by rutin, ascorbic acid, and coumaric acid in the class 5 (possibly hazardous) category. Chlorogenic acid is in the same toxicity class as artemisinin but has a higher LD_{50} than artemisinin, making it still valid for drug development. Pyrogallol, on the other hand, poses the highest risk with an LD_{50} of 300 $mg.kg^{-1}$wt in the class 3 (toxic) category, making it the most dangerous compound among the six compounds in *Holothuria atra*. Another study with those compounds from different species, such as *Camelia sinensis*[41], *Murraya koenigii*[42], *Pistacia lentiscus*[43], and *Clitoria ternatea*[44], also revealed the same toxicity classification results as this study.

CONCLUSION

The research that has been conducted describes the results, that the active compounds in *Holothuria atra* extract include chlorogenic acid, pyrogallol, rutin, coumaric acid, catechin, and ascorbic acid, which directly inhibit *Falcipain-2* that degrades hemoglobin and red blood cells in the digestive vacuole. Chlorogenic acid is the best and most stable compound with the lowest binding affinity compared to E64 (control) and forms the most hydrogen bonds with the same amino acid residue as the control. All bioactive compounds in Holothuria atra fulfill Lipinski's criteria, except chlorogenic acid and rutin. The safest bioactive compound is catechin, which has an LD50 of 10,000 mg/kg and is in toxicity class 6 (non-toxic).

CONFLICTS OF INTEREST

The authors declare no conflict of interest with respect to the research, authorship, and/or publication of this article.

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AUTHOR CONTRIBUTIONS

The authors confirm contribution to the article areas as follows ; Alifiah Wahyu: Data curation, writing and editing. Prawesty Diah Utami: Conceptualization, methodology and validation, editing, project supervision, and administration. Nita Pranitasari: Validation, editing, and project supervision.

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