

Research Article

Identification and Heme Polymerization Inhibition Activity (HPIA) Assay of Ethanolic Extract and Fraction of Temu Mangga (*Curcuma mangga* Val.) Rhizome

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Abstract: Temu mangga or *Curcuma mangga* Val. is one of Indonesian herbs from *Zingiberaceae* family that is under explored and could contain potentially active substances to serve as an antimalarial. This research intends not only to examine the antimalarial activity by means of heme polymerization inhibitor mechanism by using the ethanolic extract and fraction of *C. mangga* Val. but also to identify its compound classification. The extract of *C. mangga* Val. was obtained by Soxhlet extraction method using ethanol solvent followed by fractionation using Vacuum Liquid Chromatography with solvent sequence n-hexane, n-hexane: ethyl acetate (2:1), ethyl acetate and ethanol. The ethanol extract and ethanol fraction were analyzed by using LC-MS and GC-MS. Activities of hem polymerization inhibition showed by IC₅₀ values which were obtained from analysis of relationship between concentration sample and the percentage of inhibition using the PROBIT on statistical software. The result of HPIA assay shows that the IC₅₀ value of ethanolic extract and ethanolic fraction of *C. mangga* Val. rhizome are 2.273 and 1.479 mg/mL, respectively. It clearly shows that the heme polymerization inhibition activity of ethanolic fraction relatively better than that of ethanolic extract. Phytochemical screening determines the ethanolic extract contains saponin, terpenoid, and phenol while the ethanolic fraction contains terpenoid. Thus, terpenoid compound is presumed to be the inhibitor of heme polymerization. The results of analysis with LC-MS and GC-MS showed that the active compounds suspected to inhibit heme polymerization in ethanolic extract and fraction were (E)- λ -8 (17), 12-dien-15,16-dial and di-n-octyl phthalate, respectively. Thus, the active compounds suspected to be a new antimalarial active compound.

Keywords: antimalarial, *Curcuma mangga* Val., heme polymerization

Introduction

Malaria is still the main cause of death, especially in malaria endemic countries. There are 91 countries in the world which are malaria endemic countries, one of which is Indonesia. In 2016 there were 216 million cases of malaria worldwide, with a death rate of 445,000 people. In Indonesia, according to WHO data in 2016, 1,281 million cases of malaria and 2,200 people were reported dead with > 50% of them caused by *Plasmodium falciparum* [1]. There are five species of *Plasmodium* that infect humans, namely *P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae* and *P. knowlesi* [2]. Of the five types of *Plasmodium*, the most dangerous is *P. falciparum*, because it can cause brain complications [3].

Malaria prevention efforts have long been carried out but are still not optimal. Many factors become an obstacle in the effort. One of them is that there are malaria parasites that are resistant to commercial antimalarials available. *Plasmodium falciparum* is one of the parasites that has a high level of resistance to chloroquine which is the first and oldest antimalarial drug used since ancient times. Even resistance levels occur in the artemisinin drugs recommended by WHO for the treatment of malaria today [1], [4]. The parasitic resistance to the existing malaria drug and its spread is so fast and wide that almost all over the world encourages researchers to find new antimalarials. One source of new medicinal materials that are abundant in nature and affordable are plants.

Indonesia is a country rich in biodiversity. Indonesia's natural wealth provides opportunities to develop new medicines from natural materials, one of which is the *C. mangga* Val. rhizome. The plant belongs to the *Zingiberaceae* plant tribe which is widely used today as an ingredient of traditional medicinal herbs. *C. mangga* is known to have anti-cancer activity [5], [6]. Several previous studies found that ethanol extract of *C. mangga* rhizome has antiplasmodium activity by inhibiting the growth of *Plasmodium berghei* which is infected in male white mice at a dose of 250 mg / kg body weight with inhibitory value of 48.56% [7].

Further research on the fraction of ethanol extract of *C. mangga* has never been reported in particular the ethanol fraction by the method of inhibiting hem polymerization test. The inhibitory activity of hem polymerization in vitro by the HPIA method has been developed by [8]. By using this method, an inhibition value or IC₅₀ value of each sample is obtained. Hem polymerization inhibition value is obtained from reading the absorbance value of β -hematin crystals using ELISA reader. Inhibition of hem polymerization is obtained by comparing absorbance in the treatment group with β -hematin levels on a standard curve.

Plasmodium or malaria parasites that enter the intraerythrocytic cycle use the host hemoglobin as a source of nutrition. Nearly 75% of hemoglobin will be broken down into hem and globin. Globin is further broken down into sources of protein formation that are used as food sources with the help of protease enzymes while hem will actually inhibit the protease enzyme, spur the formation of free radicals (free hem). Free hem is toxic so that the malaria parasite is converted to hemozoin by a detoxification mechanism. Therefore, in this study identification and testing of *C. mangga*'s activity were carried out as an antimalarial agent by inhibiting hem polymerization and comparing the antimalarial activity of crude ethanol extract and ethanol fraction from the *C. mangga* Val rhizome. as an antimalarial. Crude extracts that were previously able to inhibit the growth of *Plasmodium* are possible to inhibit the growth of parasites through inhibiting the path of hem polymerization as well as the ethanol fraction. In addition, to determine the class of compounds that have the potential as antimalarial active compounds is carried out through phytochemical screening tests so that the mechanism of hem inhibition of these compounds can be known. Thus, the new potential antimalarial active compound could be found in this research.

Materials and Methods

Materials

The authenticated rhizomes of *C. mangga* were obtained from Center for Research and Development of Medicinal Plants and Traditional Medicines (B2P2TOOT), Karanganyar, Central Java, Indonesia in August 2016. Chemicals used in this research were ethanol, n-hexane, ethyl acetate, silica gel for column chromatography, chloroquine diphosphate, glacial acetic acid, hematin (ferriprotoporphyrin IX hydroxide), sodium hydroxide (NaOH), dimethyl sulfoxide (DMSO), sodium hydrogen carbonate (NaHCO₃) and distilled water. All chemicals, except distilled water (was obtained from CV. General Labora) were purchased from E. Merck.

Method of qualitative analysis

Extraction, Fractionation and Identification of Ethanolic Extract and Fraction of *C. mangga* rhizome

The dried rhizome of *C. mangga* was cut up to ± 0.2 cm. About 100 g of sample was weighed and 400 ml of ethanol was added and extracted in a Soxhlet apparatus at 70 °C. The filtrate was evaporated to dryness at 50°C in a rotary evaporator. And the above process was repeated for several times, until the sufficient amount of extract is produced. The concentrated extract of each plant was stored at 4°C until when required for use. The ethanolic extract was fractionated by using vacuum liquid chromatography on a silica gel column and eluted with n-hexane, ethyl acetate and ethanol to get the fractions. The ethanol extract and ethanol fraction were tested phytochemical screening and analyzed with LCMS and GC-MS.

Phytochemical screening

Alkaloid identification

Alkaloid identification is done by means of a sample put into a test tube and dissolved using 5% HCl as much as 5 mL. The solution was then heated for 5 minutes and 0.5 gram of NaCl was added. Supernatant and sediment are separated. The supernatant obtained was added with 3 drops of Dragendorff reagent. Samples are said to contain alkaloids when a precipitate is formed.

Flavonoid identification

Flavonoid identification is done by means of the sample dissolved using ethanol in a test tube then added with 0.5 Mg powder and 3 drops of concentrated HCl. Samples are said to contain flavanoid compounds when a red solution is formed.

Saponin identification

Saponin identification is done by means of the sample dissolved in 10 mL aquades in a test tube then shaken. Samples are said to contain saponin compounds when a stable froth is formed for 10 minutes.

Phenol identification

Identification of phenolic compounds can be done by adding 2 mL FeCl₃ to the sample in a test tube. Samples are said to contain saponin compounds when a black-green solution is formed.

Terpenoid identification

Identification of terpenoids is done by means of the sample dissolved using ethanol in a test tube then added with 0.5 mL of anhydrous acetic acid. Furthermore, it was added with 2 mL of concentrated H₂SO₄. After that, it is heated in an oven at 100 °C for 2 minutes. Samples are said to contain terpenoids when a pink-purple solution is formed.

LC-MS analysis

Samples were made with a concentration of 1 mg / mL in a methanol solvent. Samples were injected as much as 20 µL of solution into the LC-MS column with a solvent mobile phase system A (distilled water / 0.1% formic acid) and solvent B (acetonitrile / 0.1% formic acid); column RP-18, mobile phase flow rate 0.6 mL / min, ESI-MS positive ion mode detector. The elution system used is a solvent gradient at room temperature.

Heme Polymerization Inhibition Activity (HPIA) Assay

The antimalarial activity of the test compound was carried out by a heme polymerization inhibition activity assay method. This assay was carried out by the method of [8] which was modified in the concentration of hematine solution and the test compound. A total of 100 µL of 1 mM hematine solution in 0.1 M NaOH was added to the microtube, then 50 µL of test compound was added with various levels of concentration, namely 0; 0.3125; 0.625; 1.25; 2.5 and 5 mg/mL. Replication is done 3 times for each concentration. To initiate the heme polymerization reaction, 50 µL of glacial acetic acid (pH 2.6) was added into the microtube which contained a hematine solution and sample, then incubated at 37 °C for 24 hours. The positive control used was chloroquine diphosphate, while the negative control was DMSO 10%. The microtube then was centrifuged at 8000 rpm for 10 minutes. The supernatant is removed and the precipitate is washed 3 times with 200 µL of DMSO. Each washing by centrifugation at 8000 rpm for 10 minutes. The precipitate obtained was added with 200 µL of 0.1 M NaOH. Every 100 µL of solution obtained was put into a microplate 96-well and read the OD (Optical Density) value with an ELISA reader at a wavelength of 405 nm. The value of hem polymerization inhibition activity is expressed in IC₅₀, a level that can inhibit hem polymerization up to 50% compared to negative controls. IC₅₀ values can be calculated by making a concentration comparison curve with percent inhibition of the sample or by probit analysis using SPSS Version 16.0.

Results and Discussion

Extraction, Fractination and Identification of Ethanolic Extract and Fraction of *C. mangga* rhizome

C. mangga rhizomes that has been prepared was extracted by using Soxhlet extraction method and yielded 8.64% of dark brown liquid and thick texture. The obtained crude was then fractionated by VLC (Vacuum Liquid Chromatography) using several eluents including ethanol. The process produced ethanolic fractions 16.4% of yield. Then, the identification was done by using phytochemical screening (Table 1.). Phytochemical screening tests are used to identify earlier compounds obtained by their compounds.

Table 1. Phytochemical Screening Results of ethanolic extract and fraction of *C. mangga*

No.	Compound test	Samples	
		Ethanol extract	Ethanol fraction
1.	Alkaloid	-	-
2.	Terpenoid	+	+
3.	Phenolic	+	-
4.	Flavonoid	-	-
5.	Saponin	+	-

Note: (+) = compound contained in samples

The identification of the compounds in ethanol extract and fraction were performed with GC-MS. The chromatogram of each sample containing many of the mixed compounds can be seen in Figure 1. whereas the mass spectra of the target compound can be seen in Figure 2.

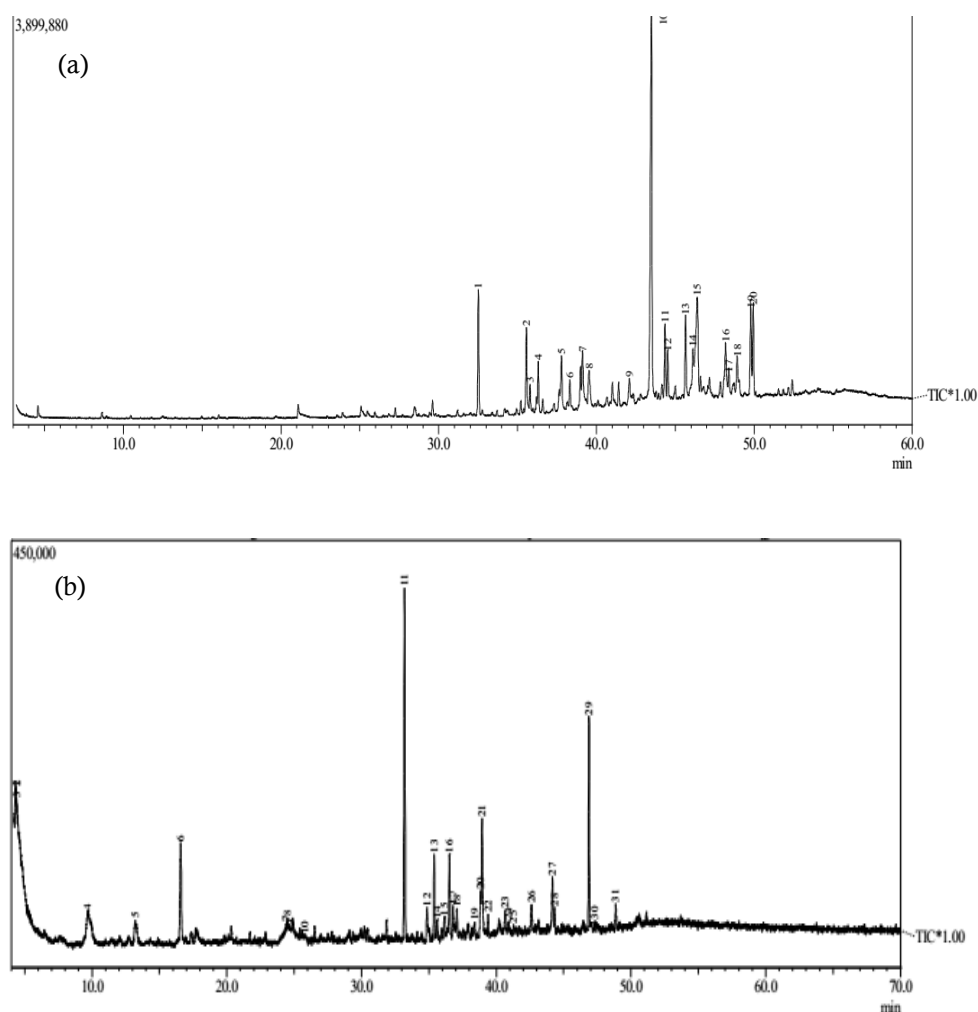


Figure 1. The gas chromatography–mass spectrometry (GC-MS) chromatograph of (a) ethanol extract and (b) ethanol fraction of *C. mangga*

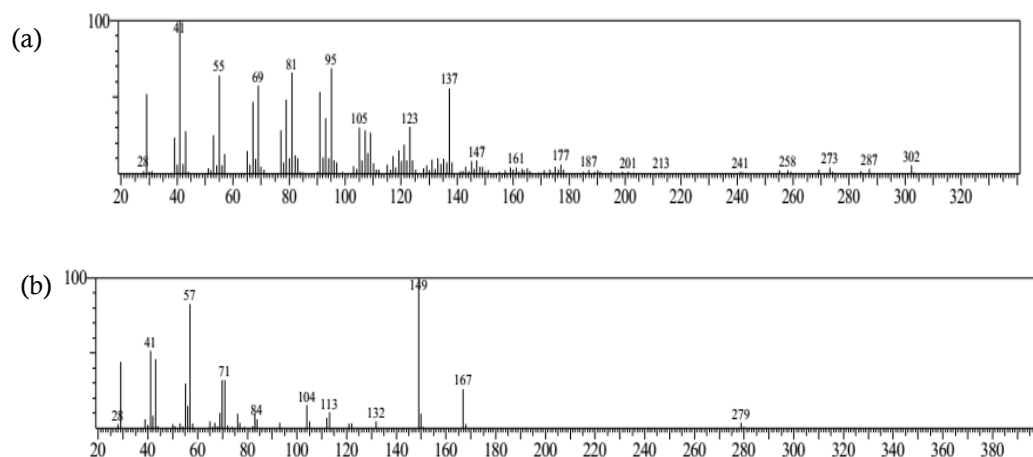


Figure 2. The Gas chromatography–mass spectrometry (GC-MS) mass spectrum of major compound in (a) ethanol extract and (b) ethanol fraction

Based on the mass spectrum results of the ethanol extract shown in Figure 2, the compound contained in the ethanol extract has a molecular weight of 302 m/z. Compounds with a molecular weight of 302 m/z have a base peak of 41 m/z. The compound is (E)-labda-8 (17), 12-dien-15,16-dial. The fragmentation pattern of (E)-labda-8 (17), 12-dien-15,16-dial showed in Figure 3.

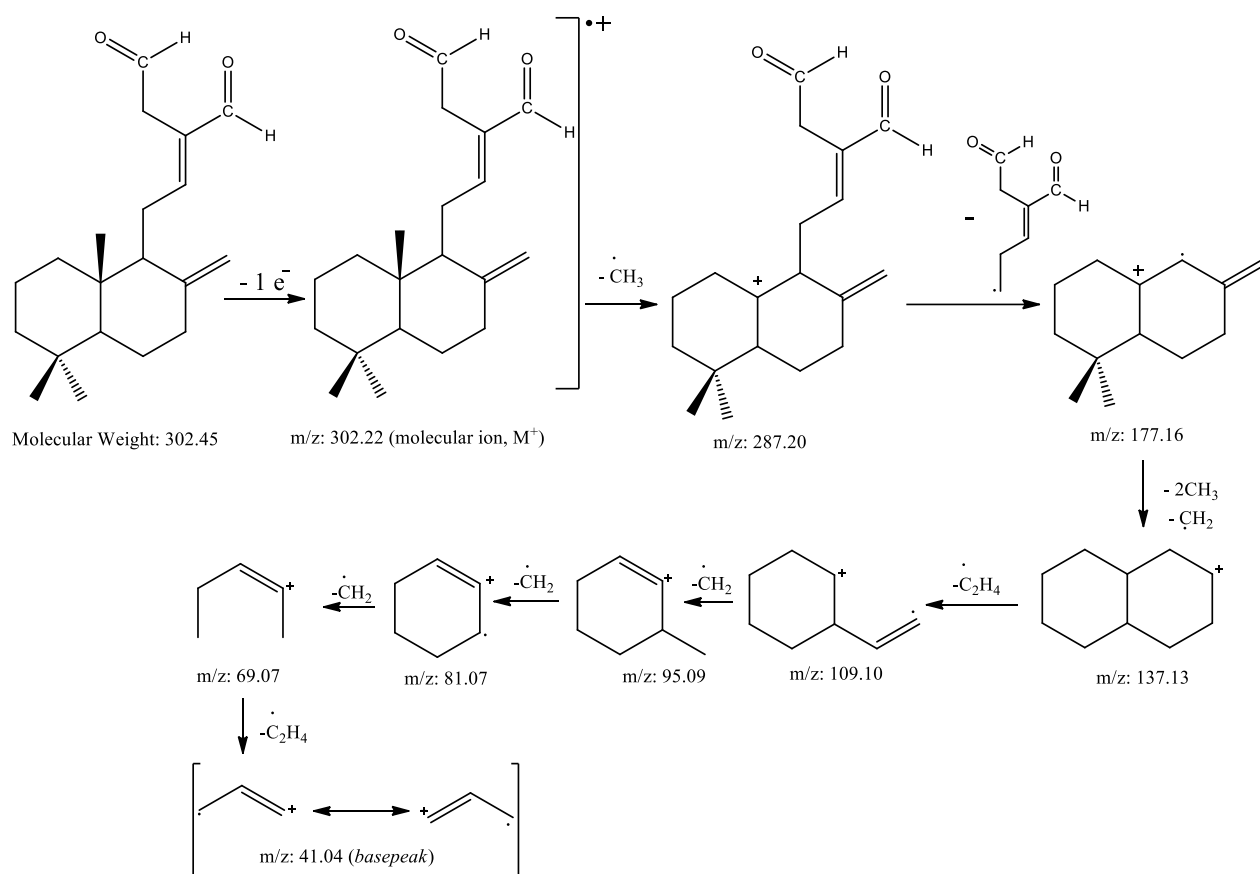


Figure 3. The fragmentation pattern of (E)-labda-8 (17), 12-dien-15,16-dial

[9] states and has identified (E)-labda-8 (17), 12-dien-15,16-dial in *C. mangga* Val's methanol extract, stating that the compound has a molecular weight of 302 m/z. This was also proven by calculating the molecular weight of the compound using the 2016 version of the Chemical Draw software application, obtained a molecular weight of 302.45 m/z. [10] states that the *C. mangga* rhizome contains compounds (E)-labda-8 (17), 12-dien-15,16-dial which belongs to the "labdane diterpene glucoside" group. This is also consistent with the phytochemical screening results shown in Table 1, where ethanol extract contained terpenoids.

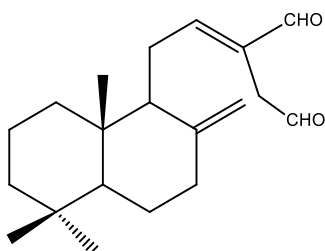


Figure 4. Structure of (E)-labda-8 (17), 12-dien-15,16-dial compound

Analysis using GC-MS on ethanol fraction obtained there are two dominant peaks. Based on the mass spectra produced, it can be seen that the compound included in the active substance at peak number 29 with a retention time of 46.89 minutes with an abundance of 10.89% is shown in Figure 2 b. The mass spectrum analysis is based on the degree of similarity between the sample mass spectrum and the mass spectrum of the library. The compound contained in the ethanol fraction has a molecular weight of 390 m/z. Compounds with a molecular weight of 390 m/z have a base peak of 149 m/z and a similarity level of 91 presumably is di-n-octyl phthalate.

HPIA Assay

Testing the antimalarial activity of ethanol extract and ethanol fraction was done by testing the inhibition of hem polymerization. *Plasmodium* or malaria parasites that enter the intraerythrocytic cycle use the host hemoglobin as a source of nutrition. Nearly 75% of hemoglobin will be broken down into hem and globin. Globin is further broken down into sources of protein formation that are used as food sources with the help of protease enzymes. While hem will actually inhibit the protease enzyme, spur the formation of free radicals (free hem). Free hem is toxic so that the malaria parasite is converted to hemozoin by a detoxification mechanism.

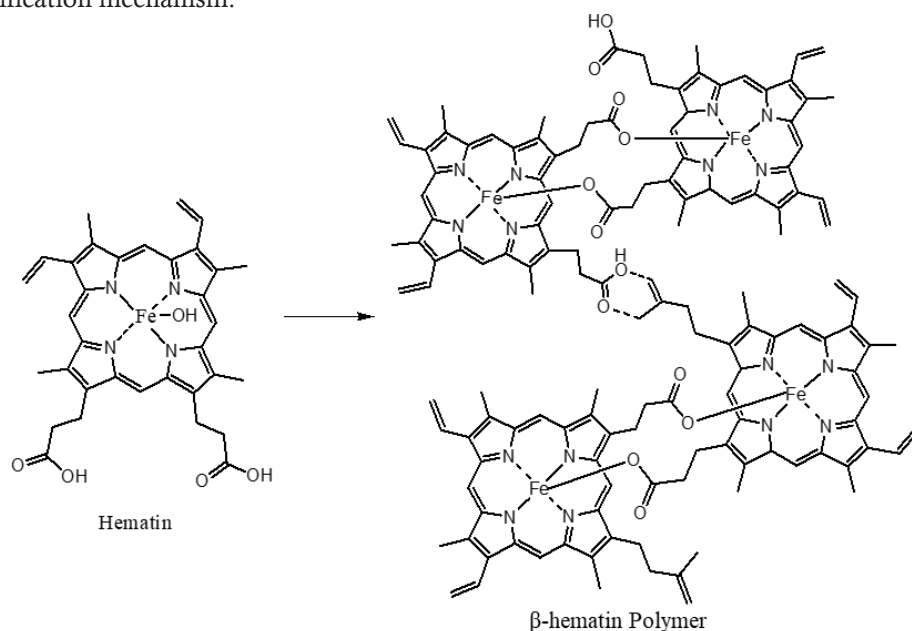


Figure 5. Hem Polymerization Reaction from Hematin to β -hematin

Hemozoin as malaria pigment is a compound that is difficult to dissolve. Hemozoin formation can be modeled in vitro as was done in this study. Hemozoin is identical to the β -hematin polymer formed from

hematin in an acidic atmosphere and hematin is identical to hem [11], [12]. Therefore, to find out the antimalarial activity of a sample can be done in vitro using the process of hem polymerization inhibition. Hem polymerization reaction from hematin to β -hematin can be seen in Figure 5.

The hem polymerization inhibition activity test of the samples occurs spontaneously by the addition of the sample and glacial acetic acid into the hematin solution. The test material used was ethanol extract and ethanol fraction of *C. mangga* rhizome. This study uses 10% dimethyl sulfoxide (DMSO) as a negative control and chloroquine diphosphate as a positive control whose antimalarial activity was previously known through inhibition of hem polymerization. Concentration of sample and negative control was made by dissolving the test material with DMSO. In this study 10% DMSO was used as a solvent for the sample. The results of previous studies stated that the addition of DMSO with a concentration of 0.5-10% had a small inhibitory value even it could be said to have no inhibitory activity for hem polymerization [13]; [8].

Formation of β -hematin crystals through inhibition of hem polymerization begins with the addition of glacial acetic acid (pH 2.6) and incubated for 24 hours at 37 °C and pH 3.0-5.5 because at that temperature and time, β -hematin crystals formed optimally. Glacial acetic acid is used as a regulator of acidity. It is adjusted to a pH in the digestion vacuole, which is where the process of reduction of the toxicity of free heme by the malaria parasite.

Acidic atmosphere is the optimal pH in converting hematin to changing hem to hemozoin in acidic conditions at pH 4.9-5.9. The process of β -hematin formation will be preceded by the formation of amorphous hem deposits, and is followed by a slow conversion to β -hematin crystalline [14]. β -hematin formed can be separated from hematin which does not undergo polymerization by washing using DMSO. In this study DMSO was used as a washing solution because it could not dissolve β -hematin crystals but dissolve hematin which did not undergo polymerization alone.

The results of the hem polymerization inhibition test on ethanol extract and the ethanol fraction of *C. mangga* rhizome are shown in Table 2. The amount of β -hematin crystals that are read will be inversely proportional to the antimalarial agents inhibiting the hem polymerization in this case in the form of sample test material. IC_{50} values were obtained using probit analysis. IC_{50} is the concentration of test material needed to inhibit 50% of parasitic growth. The smaller the concentration needed, the better the activity of these compounds as antimalarials.

Table 2. Result of Heme Polymerization Inhibition Assay of ethanolic extract and fraction of *C. mangga* rhizome

Samples	Concentration (mg/mL)	Average dose of hemozoin (mM)	Average percent of Inhibition	IC_{50} (mg/mL)
Ethanolic extract	5	36.46 ± 2.67	79.39 ± 1.51	2.723
	2.5	109.78 ± 2.48	37.95 ± 1.40	
	1.25	137.70 ± 7.37	22.17 ± 4.17	
	0.62	165.24 ± 11.93	6.60 ± 6.74	
	0.31	152.93 ± 14.11	13.56 ± 7.97	
Ethanolic fraction	5	31.28 ± 4.44	82.32 ± 2.51	1.479
	2.5	84.46 ± 8.86	52.26 ± 5.01	
	1.25	103.79 ± 2.91	41.33 ± 1.64	
	0.62	118.07 ± 11.70	33.26 ± 6.61	
	0.31	133.44 ± 9.78	24.58 ± 5.53	
Chloroquine diphosphate	5	109.03 ± 9.58	31.80 ± 5.99	19.760
	2.5	179.78 ± 16.87	-12.46 ± 10.56	
	1.25	159.65 ± 49.72	0.13 ± 31.10	
	0.62	141.66 ± 30.42	11.39 ± 19.03	
	0.31	152.84 ± 65.08	4.39 ± 40.71	

Based on the results of hem polymerization inhibition activity assay shown in Table 2, it shows that the ethanol extract and fraction from *C. mangga* rhizome has IC_{50} values of 2.723 and 1.479 mg/mL, respectively, while the positive control of chloroquine in this study had an IC_{50} value of 19.760 mg/mL. When compared with the positive control of the research results and some supporting literature, ethanol extract and ethanol fraction have better activity than chloroquine. Meanwhile, according to [15] if IC_{50} hem polymerization inhibitor activity of a compound is more than 12 mg / mL (chloroquine sulfate 37.5 mM) then it can be categorized as not having hem polymerization inhibitor activity. Based on the results of hem

polymerization inhibition test and supporting literature, it can be concluded that ethanol extract and fraction of *C. mangga* have activity as hem polymerization inhibitors.

Hem polymerization inhibitory activity is actually the work of one or two mechanisms, namely (1) interaction occurs between terpenoid compounds, phenols and sterols with electronic systems of hem, (2) these extracts consist of compounds that have hydroxyl groups that can bind to hem iron ions [8]. Thus, the mechanism of action of ethanol extract and ethanol fraction of *C. mangga* rhizome Val. in inhibiting hem polymerization by interacting terpenoid compounds, phenols and sterols with hem electronic systems, but does not rule out the existence of other groups of compounds that are able to inhibit malaria growth by inhibiting hem polymerization.

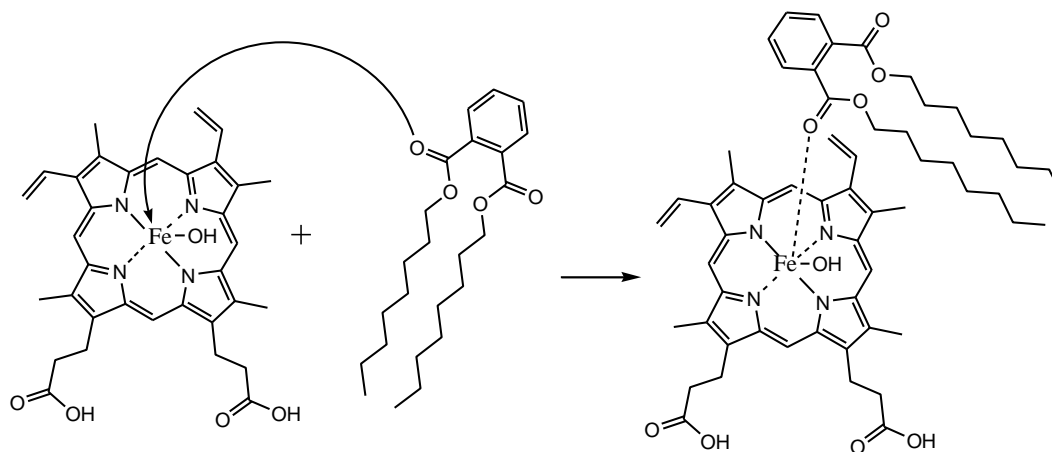


Figure 6. Mechanism of Heme Polymerization Inhibition of di-n-octyl phthalate

Hemoglobin degradation occurs due to the release of Fe^{2+} -hematin oxidized to Fe^{3+} -hematin, and then precipitated in the food vacuole to form hemozoin (malaria pigment). Inhibition of hemozoin formation is one mechanism of malaria parasitic activity. One mechanism in vitro that is used is the inhibition of hem polymerization [16]. The proposed mechanism of inhibition of hem polymerization can be seen in Figure 13. which is the mechanism of the active compound in-n-octyl phthalate inhibiting hemozoin formation.

[17] have conducted in vivo antimalarial activity test of *Alstonia boonei* bark extract, it is known that the di-n-octyl phthalate compound is the dominant compound in the water fraction. This compound is thought to be an active compound that can inhibit the growth of parasitemia. This inhibitory mechanism can occur due to the entry of antimalarial agents into the parasitic food vacuole and then interacts with Fe^{2+} -hematin [18]. Interaction begins with the formation of antimalarial chelate between positive groups (central atoms) found in hematin and negative groups (ligands) found in compounds. The presence of coordinating covalent bonds between Fe^{3+} -hematine with free electron pairs can inhibit the formation of hematine crystals (hemozoin) [19].

Conclusion

Extract and fraction of *C.mangga* contained (E) -labda-8 (17), 12-dien-15,16-dial compound. The result of HPIA assay shows that the IC_{50} values of ethanolic extract and ethanolic fraction of *C. mangga* Val. rhizome are 2.273 and 1.479 mg/mL, respectively. It clearly shows that the heme polymerization inhibition activity of ethanolic fraction relatively better than that of ethanolic extract. Phytochemical screening determines the terpenoid compound is presumed to be the inhibitor of heme polymerization. The results of analysis with LC-MS and GC-MS showed that the active compounds suspected to inhibit heme polymerization in ethanolic extract and fraction were (E) -labda-8 (17), 12-dien-15,16-dial and di-n-octyl phthalate, respectively. Thus, the active compounds suspected to be a new antimalarial active compound.

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