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Research Article
Antimalarial Activity of the Fractions from Ethanol Extract of *Strychnos ligustrina* Blume. Wood

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Abstract

Background and Objective: This study identify the fraction contents from ethanol extract of *Strychnos ligustrina* Blume wood, their antimalarial activities and chemical compound of the antimalarial active fraction. **Methodology:** The ethanol extract was obtained from maceration with n-hexane, ethyl acetate and ethanol successively. The ethanol extract was fractionated using vacuum liquid chromatography column with combination of chloroform and methanol as eluent. These fractions were tested for their antimalarial properties by using *in vitro* against *Plasmodium falciparum*. The active antimalarial fraction was then chemically analyzed by using gas chromatography mass spectrometry instrument. **Results:** The fractionation of ethanol extract resulted in six fractions, namely F1, F2, F3, F4, F5 and F6 with fraction contents at 2.48, 0.89, 14.28, 22.34, 25.57 and 30.34%, respectively. The antimalarial bioassay test showed that F3 and F4 were very active with IC_{50} (1.99 and 0.39 μg mL^{-1}, respectively) F1 was active with IC_{50} (81.04 μg mL^{-1}) while, F2, F5 and F6 were inactive with IC_{50} (1581.60, 1123.22 and 81.04 μg mL^{-1}, respectively). Gas chromatography mass spectrometry instrument detected the F3 contains alkaloids such as (phenylbenzo[f][1,7] naphthyridin-5-one (11.67%), 4-methyl-5-[3-trifluoromethylphenoxy]-6-methoxy-8-nitroquinoline (5.21%) and phenolic compounds (coniferol 8.10%). The F4 is dominated by furan compounds (hydroxymethyl furfural 19.43%), phenolic (laminitol 19.43%), fatty acid (9,11-octadecadiynoic acid, 8-hydroxy-methylester 6.34%) and alkaloid (brucine 6.13%). **Conclusion:** Extractive substances of *S. ligustrina* wood were very potential sources for natural antimalarial drugs. Fractionation ethanol extract of *S. ligustrina* wood produced fraction F3 and F4 were very active in inhibiting the growth of *P. falciparum.* These results strongly suggested that F3 and F4 of *S. ligustrina* wood were potential sources for antimalarial agents. For future development, it is neccesary for further studies to obtain the antimalarial active compounds from fraction F3 and F4 as an alternative new malaria drugs as well as material alternative drug combinations with artemisinin to get the optimal treatment of malarial.

Key words: Antimalarial, ethanol extract, *in vitro* bioassay test, *Plasmodium falciparum*, *Strychnos ligustrina* Blume

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Malaria is one of the contagious diseases that still become a major health problem in the world including Indonesia. This disease is caused by parasites (protozoa) of the genus *Plasmodium* and can be transmitted to humans through the bite of Anopheles mosquito. *Plasmodium* species that capable of infecting humans are *Plasmodium falciparum*, *P. vivax*, *P. ovale* and *P. malariae*[1]. Of the four species of *Plasmodium* that infect humans, *P. falciparum* is the most dangerous parasite plasmodium species and most often infects people in Indonesia[2]. Malaria infects an estimated of 300-500 million people worldwide and resulted in 2.5-2.7 million sufferers die[3] each year. This disease is still endemic in a large part of Indonesian territory including East Nusa Tenggara (NTT) whose endemicity rank is 3rd out of 33 provinces[4].

An increase in the resistance of *P. falciparum* to conventional treatments such as chloroquin is another worldwide problem while, drug alternative to substitute chloroquine is very limited and causes side effects[1]. One of substitute drugs chloroquine is artemisinin namely sesquiterpene lactone compound which were isolated from *Artemisia annua* plants[5]. However, artemisinin has side effects and has been occurring parasite resistance to artemisinin in Southeast Asia including Cambodia, Myanmar, Thailand and Vietnam[6]. Therefore, treatment of malaria which were recommended by the WHO is the use of drugs Artemisinine Based Combination (ACT). Antimalarial drug combinations with artemisinin and its derivatives provide therapeutic effectiveness upto 100% for 3 days of treatment[5]. The side effects of anti-malarial drug and the increasing resistance of *plasmodium* especially to artemisinin have encouraged studyers to explore and identify new antimalarial drugs. One is through the exploration of bioactive compounds from natural medicinal materials, particularly herbs that have traditionally been used to treat malaria.

The active compounds that is contained in extractive of anti-malarial medicinal plant was very potential to be developed as an antimalarial drug ingredient that can be combined with artemisinin. This combination therapy has the advantage that able to improve the effectiveness of the bioactive compound also have the potential to slow the parasite resistance to antimalarial drugs standard.

One of the Indonesian plants which are potential to be developed as a natural anti-malarial drugs is *Strychnos ligustrina* Blume. Until now, there were only few studies and scientific evidence about the activities of the bioactive compounds of *S. ligustrina* as an antimalarial drug while, the study on anti-malarial active compounds obtained from the species *S. usambarensis* and *S. icaja* was conducted by Frederich *et al.*[7]. The active substance from *Strychnos* plants that have antimalarial against *P. falciparum* is the alkaloid compound such as strychnopentamine, isostrychnopentamine (IC_{50} value of 0,15 μM) and dihydrousambarensin (IC_{50} value of 0.03 μM), extracted from the root bark of *S. usambarensis*[7].

In addition, study on the utilization of crude extract of *S. ligustrina* wood as an anti-malarial is also very limited. Huda[8] reported that the *in vivo* testing of the *S. ligustrina* wood water extract with a 50% effective dose (ED_{50}) of 0.45 mg kg^{-1} has a great potential as an antimalarial and *S. ligustrina* water extract *in vitro* can inhibit parasite growth[9] upto 100%. Philippe *et al.*[10] reported another species of *Strychnos* that is the ethanol extract of *S. variabilis* roots which also has a great potential as antimalarial with IC_{50} value of 2 μg mL^{-1}. The active substance in *S. ligustrina* wood is strychnine and brucine[11] as well as ester quinat acid[12]. The *S. ligustrina* wood also contains loganin[13] but its antimalarial activities has not yet been tested. Syafii *et al.*[14] reported that the yield of n-hexane, ethyl acetate and ethanol extracts from the solvent extraction with maceration method of *S. ligustrina* wood by stratified polarity were 0.55, 1.24 and 4.11%, respectively. Antimalarial activity of the ethanol extract by using *in vitro* tests against *P. falciparum* was the most active with IC_{50} value of 3.09 μg mL^{-1} while, the ethyl acetate and n-hexane extracts were classified as inactive (IC_{50} value of 889.30 and 81.38 μg mL^{-1}, respectively). Based on the above previous study, the objectives of this study were to determine the fraction contents from ethanol extract of *S. ligustrina* Blume wood, their antimalarial activities and chemical compounds of the antimalarial active fraction, using a gas chromatography-mass spectrometry (GCMS).

The results are expected to be a reference in advanced study to be able to get new antimalarial active compounds that can be combined with artemisinin to produce new malaria drug combinations that have the effect of antimalarial better than artemisinin monotherapy.

MATERIALS AND METHODS

Ethanol extract preparation: The wood of *S. ligustrina* derived from natural forests in the region of Lombok, West Nusa Tenggara, Indonesia. Wood was made into woodmeal using Willey grinder then filtered to obtained woodmeals of 40-60 mesh size. The extraction method was based upon Syafii *et al.*[14] that is maceration using the solvent by stratified polarity carried out at room temperature. The solvents used include n-hexane, ethyl acetate and ethanol with woodmeal and solvent ratio was 1:3. A total of ±2000 g samples of the woodmeal were extracted with n-hexane for 24 h. Extraction is conducted repeatedly until the n-hexane extract was colorless. Furthermore, the residue was multilevel extracted with ethyl acetate and ethanol. Extract n-hexane, ethyl acetate and ethanol were obtained then concentrated at a temperature of 40-50°C in a rotary evaporator to ±100 mL and dried in the oven at 40°C for *in vitro* antimalarial activity testing.

Fractionation of ethanol extract: About 5 g of concentrated ethanol extract was dissolved in ethanol and fractionated using vacuum liquid chromatography column. Packaging was done using a 150 g column of silica G 7731. Best eluent (chloroform and methanol) were used as mobile phase with a gradient system. Eluate was collected in several bottles of 90 mL, each of which was given a number then tested by TLC. The spots separated in TLC was observed with 254 nm UV light. The Rf and a eluate which have the same pattern spots appearance on TLC are combined as one fraction then concentrated and tested for their anti-malaria activities to obtain the most active fraction.

Antimalarial activity test: The testing procedure referred to the methods used by Widyawaruyanti *et al.*[15]. *Plasmodium falciparum* 3D7 strain was used to test the antimalarial activity. A total of 10 mg of each fraction was dissolved in 100 mL of dimethyl sulfoxide (DMSO) as stock solution of the test sample. Next, 10 mL of stock solution of the test sample was added to 500 mL of test medium. Complete medium and test sample solution was put into the wells of microtiter plates (24 holes) and made into serial dilutions so that the final concentration in microtiter wells were at 0.01, 0.1, 1, 10 and 100 μg mL^{-1}. After that 500 mL suspension parasetimia parasite with 1 and 5% hematocrit was added into each microtiter wells and then incubated in a candle jar for 48 h at 37°C. Cultures that were incubated were subsequently harvested and made into blood thin layer preparations with 20% of Giemsa staining. Culture was left for 20 min then washed with water and dried. Tests were carried out for 2 times of replication. Then, the percentage of parasetimia and percent growth inhibition were calculated by counting the number of *P. falciparum* infected by erythrocytes per 5000 erythrocytes under the microscope.

Parasitaemia percentage is calculated by the formula:

$$\text{Parasitemia (\%)} = \frac{\sum \text{infected erythrocytes}}{5000 \text{ erythrocytes}} \times 100$$

Percentage inhibition of growth of *P. falciparum* is calculated using the formula:

$$\text{Inhibition (\%)} = \frac{Xp}{Xk} \times 100 \text{ (\%)}$$

Where:
Xp = Percentage of growth parasitaemia in treatment
Xk = Percentage of growth parasitaemia in negative control

Antimalarial activity was determined from IC_{50} value of the fraction that is the fraction of the concentration in which the percentage of inhibition of parasite growth was 50%. The smaller the IC_{50} value means the higher the antimalarial activity. To determine the IC_{50} value, probit analysis was used to make the curve relationship between probit (probability unit) percentage inhibition and the logarithmic levels.

Chemical components analysis of the most antimalarial active fraction: Chemical components of the most active fractions were detected using GC-MS Agilent technologies 6890N series. Software data analysis GC-MS were used in data processing. Separation of compounds and quantitative analysis performed on GCMS components were conducted by a capillary column with a diameter of 0.25 mm and a length of 60 m with a starting temperature of 40°C, rising by 15°C min^{-1} until temperature reached 290°C and ending time was 10 min. The identification of the compounds was performed by comparing the data in the mass spectrum with existing data in the 9th WILEY library.

RESULTS AND DISCUSSION

Fractionation of the ethanol extract using vacuum liquid chromatography produced 6 fractions (Table 1). Fraction grouping was based on the TLC analysis using chloroform and methanol (8.5:1.5) as the best eluent.

Based on Table 1, the yield of fractions obtained ranged from 0.89-30.34%. Fraction number 5 had the highest yield of 30.34% followed by fraction 6, 4, 3 and 1 and the smallest yield was fraction 1 (2:38%). Although, the yield of fraction 5 and 6 were relatively dominant, they were not a single compound yet, because the number of spots on TLC analysis was more

Table 1: Yield and characteristics of fractions

Fraction	Weight (g)	Yield (%)*	Characteristics extracts of fraction	Rf in TLC
F1	0.119	2.3	Solids, whitish yellow	0.8, 0.9
F2	0.045	0.1	Solids, whitish yellow	0.7, 0.6
F3	0.713	14.2	Solids, yellow	0.6, 0.2
F4	1.117	22.3	Solids, yellow	0.6, 0.5
F5	1.517	30.3	Solids, yellowish brown	0.6, 0.5, 0.4
F6	1.278	25.5	Solids, brown	0.5, 0.3
Total	4.789			

*Based on the dry weight of ethanol extract (5 g)

Table 2: IC_{50} values of the fraction from *S. ligustrina* Blume wood extracts

Fraction	IC_{50} (μg mL^{-1})	Antimalarial activity*
F1	8.15	Active
F2	1581.60	Inactive
F3	1.99	Very active
F4	0.39	Very active
F5	1123.22	Inactive
F6	81.04	Inactive
Artemisinin	$<1.0\times10^{-4}$	Very active

Based on Phillipe *et al.*[10]

than one. The existence of multiple spots on the plate indicated some compounds. Fractionations conducted by Juita[16] to test ethanol extract antimalarial activity of the leaves of *Tithonia diversifolia* Hemsl. against *P. berghei* using the vacuum liquid chromatography produced 5 fractions that do not have a single compound. The weakness of this method was the inability to get a single compound as the eluate was forced down so that the separation of the compound was less optimal. Table 1 shows that the total weight of the fraction was 4.789 g due to the fact that during fractionation there were samples which were not carried away by the solvent. Futhermore, the color of each fraction of *S. ligustrina* wood ethanol extract was different visually. This indicated that the compounds content of each fraction varied in types and compositions. Lubis[17] states there were differences in the color of the fractions produced from ethyl acetate fraction *S. ligustrina* wood that is solid dark yellow.

The results showed that there was a positive correlation between the concentrations of fractions with the percentage of *P. falciparum* parasite growth inhibition (Fig. 1). The test results of *in vitro* antimalarial activity showed that all fractions of *S. ligustrina* wood ethanol extract had the ability to inhibit the growth of *P. falciparum* in the range of 0-100%.

Table 2 shows the IC_{50} values of all six fractions the *S. ligustrina* wood ethanol extract. The different value indicated different antimalarial activity.

Fraction number 3 and 4 had a very active antimalarial activity, fraction 1 was classified as active whereas fractions 2, 5 and 6 were classified as inactive. Fraction 4 was the most active fraction with IC_{50} values of 0.386 μg mL^{-1} and yield fractions of 22.34%. This shows that fraction 4 is a very prospective fraction to be developed as an antimalarial agent. Philippe *et al.*[10] stated that extracts that have very active antimalarial activity with IC_{50} values $\leq$5 μg mL^{-1} were classified into active, less active and inactive when the IC_{50} values were 5-15, 15-30 and >30 μg mL^{-1}, respectively.

Artemisinin as a positive control has very active antimalarial activity with IC50 values $<0.1\times10^{-4}$ μg mL^{-1}. Its

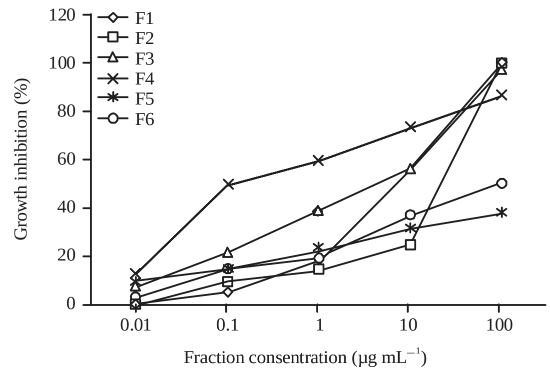

Fig. 1: Relations of concentration of *S. ligustrina* wood ethanol extract fraction with the percentage of *P. falciparum* parasite growth inhibition

antimalarial activities were much higher than fractions 3 and 4. The reason for this was that artemisinin was pure compound while, fractions 3 and 4 still contained many compounds as shown by the number of spots on TLC which are more than one (Table 1).

Analysis of the chemical components of the two most active fractions by GC-MS found 77 compounds in fraction 3 (Table 3) and 61 compounds in fraction 4 (Table 4).

The results of GC-MS analysis showed that fraction 3 was dominated by the alkaloid phenylbenzo [f][1,7] naphthyridin-5-one (11.67%), phenolic coniferol (8.10%), alkaloids 4-methyl-5-[3-trifluoromethylphenoxy]-6-methoxy-8-nitroquinoline (5.21%) and other compounds with relatively small concentration (0.37-1.36%). References regarding to the compounds detected in fractions 3 show that alkaloid strychnine and anthraquinone catenarin were suspected t to contribute to the antimalarial activity of fractions 3. Frederich *et al.*[7] reported that the strychnine compound isolated from the extract of the roots of *S. isaja* were found to have antimalarial activity with IC_{50} value of 8.5 mg mL^{-1}. However, these compounds had a relatively small concentrations in fractions 3 with its relative concentration of 1.17%. Osman *et al.*[25] reported that anthraquinone, dichloromethane extract from the *Rennellia elliptica* roots Korth has antimalarial activity against *P. falciparum* with IC_{50} 4.04 μg mL^{-1}. The absence of a report on antimalarial activity of other dominant compounds in fractions 3 was of great concern to be studied in further study to isolate and test the antimalarial activity of isolates dominant compound in fractions 3.

Analysis of the chemical components by GC-MS of the most active fraction (fraction 4) shows compounds content as presented in Table 4.

Table 3: Chemical compounds in fractions 3 based on GCMS analysis

Compounds[1]	Relative consentration (%)[2]	Bioactivity[3]
Phenylbenzo[f][1,7]naphthyridin-5-one	11.67	Antiviral[18]
Coniferol	8.10	Antibacterial[19]
4-Methyl-5-[3-trifluoromethylphenoxy]-6-methoxy-8-nitroquinoline	5.21	Antibacterial[20]
Hydroxymethylfurfural	1.36	Antimicrobial[20]
Benzoic acid, 4-methyl, methyl ester 1.27 Antibacterial1	1.27	Antibacterial[19]
Strychnine	1.17	Antimalarial[7]
Phenol, 4-ethenyl-2-methoxy	0.91	Antibacterial[21]
2,4-dimethoxyphenol	0.79	Antibacterial, antioxidant[22]
Guaiacol	0.61	Antioxidant[23]
Isoeugenol	0.59	Antioxidant[24]
Hexadecanoic acid	0.58	Antibacterial[22]
Vanillin	0.41	Antioxidant[23]
Catenarin	0.37	Antimalarial[25], Antibacterial[26]

[1]Based on GC-MS (Gas chromatography mass spectrometry) analysis, 2: The Relative concentrations of77 compounds detected and 3: Based on the study literature

Table 4: Chemical compounds in fractions 4 based on GC-MS analysis

Compounds[1]	Relative consentration (%)[2]	Bioactivity[3]
Hydroxymethylfurfural	19.43	Antioxidant[27]
Laminitol	19.43	Antibacterial[28]
9,11-octadecadiynoic acid, 8-hydroxy, methyl ester	6.34	Antibacterial[19]
Brucine	6.13	Antiinflammatory and analgesic[6]
Phyllamyricin A	3.18	Antimicrobial[29]
2-methoxyhydroquinone	1.35	Antimicrobial[29]
Anthracene, 9,10-bis(2-methoxyphenyl	1.25	Antibacterial[26]
2-[3-Hydroxy-4-methoxyphenyl]-semicarbazide	0.44	Antioxidant[21]

1: Based on GC-MS (Gas chromatography mass spectrometry) analysis, 2: The Relative concentrations of 61 compounds detected and 3: Based on the study literature

The compounds in fraction 4 contain a variety of classes of compounds such as furans, phenolics and alkaloids. Fraction 4 contains compounds which include furan compounds hydroxymethyl furfural (19.43%), phenolic laminitol (19.43%), fatty acid 9,11-octadecadiynoic acid, 8-hydroxy, methyl ester (6.34%), alkaloid brucine (6.13%) and other phenolic compounds with relatively small concentration (0.44-3.18%) such as phyllamyricin A, 2-methoxyhidroquinon, antrasen and 2-[3-hydroxy-4-methoxyphenyl]-semicarbazide. Brucine is a compound with the third largest relative concentration found in the ethanol extract fraction 4 from *S. ligustrina* wood. Some of the active substances found in *S. ligustrina* wood was strychnine and brucine[11] however, there has been no report about brucine antimalarial activity. Yin *et al.*[30] succeeded in isolating brucine in *Strychnos nuxvomica* plant which has anti-inflammatory and analgesic activity. Based on references studyes have not found reports on antimalarial activity of compounds detected in the fraction 4.

CONCLUSION

Extractive substances of *S. ligustrina* wood were very potential sources for new natural antimalarial drugs. This study showed that fractionation ethanol extract of *S. ligustrina wood* resulted in six fractions with extractive content namely, F1 (2.38%), F2 (0.89%), F3 (14.28%), F4 (22.34%), F5 (30.34%) and F6 (25.57%), respectively. The antimalarial bioassay test showed that F3 and F4 were very active with IC_{50} value of 1.99 and 0.39 µg mL^{-1}, respectively F1 was active with IC_{50} value of 81.04 µg mL^{-1}, while F2, F5 and F6 were inactive with IC_{50} value of 1581.60, 1123.22 and 81.04 µ gmL^{-1}, respectively). Chemical determination of F3 by using GC-MS detected the presence of dominant compounds such as alkaloids phenylbenzo[f][1,7]naphthyridin-5-one(11.67%), 4-methyl-5-[3-trifluoromethyl phenoxy]-6-methoxy-8-nitroquinoline (5.21%) and phenolic compounds coniferol (8.10%) which suspected having antimalarial activity in F3. The GCMS analysis in F4 detected the presence of a dominant compounds such as furan compounds hydroxymethyl furfural (19.43%), phenolic laminitol (19.43%), fatty acid 9,11-octadecadiynoic acid, 8-hydroxy, methyl ester (6:34%) and alkaloid brucine (6.13%). These results strongly suggested F3 and F4 were potential fraction to be developed as antimalarial agents. However, necessary to conduct further study to isolate and identify the pure compound antimalarial in F3 and F4 from the ethanol extract of *S. ligustrina* wood. For future development, the antimalarial active compounds from fraction F3 and F4 hopefully can be use as an alternative new malaria drugs and material alternative drug combinations with artemisinin to get the optimal treatment of malarial.

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