

Antimalarial Activity and Phytochemical Profile of Ethanolic and Aqueous Extracts of Bidara Laut (*Strychnos ligustrina* Blum) Wood¹

Harisyah MANURUNG² · Rita Kartika SARI^{2,†} · Wasrin SYAFII² ·
Umi CAHYANINGSIH³ · Wiwied EKASARI⁴

ABSTRACT

This study aimed to determine the antimalarial effect of the *Strychnos ligustrina* (SLW) wood extracts and to analyze its phytochemicals. The SLW powder samples were macerated with 100% ethanol (E100), 75% ethanol (E75), 50% ethanol (E50), 25% ethanol (E25), and aqueous (A100). The extracts were analyzed by LCMS/MS, and its *in-vitro* antimalarial activity was tested with *Plasmodium falciparum*. The results showed that the extract yields of E100, E75, E50, E25, and A100 were 4.3, 5.2, 5.3, 4.7, and 3.6%, respectively. The antimalarial activities of the A100, E25, E50, and E75 extracts were classified as active with IC₅₀ values of 38.6, 42.6, 42.9, and 43.7 µg/mL, respectively. But, the antimalarial activity of the E100 extract was classified as slightly active with IC₅₀ values of 87.4 µg/mL. The dominant compounds contained in the extracts of A100, E25, E50, E75, and E100 was the alkaloid compound, namely brucine with relative concentrations of 24.96, 24.55, 21.33, 11.79, and 11.62%, respectively.

Keywords: antimalarial activities, aqueous, bidara laut (*Strychnos ligustrina* Blum), ethanol, phytochemical profile, *Plasmodium falciparum*

1. INTRODUCTION

Indonesia is known as the second mega-biodiversity country in the world because of its immense biodiversity. The natural resources of plants in Indonesia include 30,000 plant species from a total of 40,000 plant species in the world and 940 of them are medicinal plants (Dephut, 2010). One of the plants that can be

developed as a medicinal plant is *S. ligustrina*. Empirically, *S. ligustrina* is used by the Indonesian people, especially from West Nusa Tenggara (NTB) as an antimalarial drug (Setiawan *et al.*, 2014). Several studies showed that *S. ligustrina* wood has a potential active ingredient for antimalarial drugs (Huda, 2006, Syafii *et al.*, 2016a, and Syafii *et al.*, 2016b).

Malaria is one of the infectious diseases which is

¹ Date Received January 21, 2019, Date Accepted August 10, 2019

² Department of Forest Products, Faculty of Forestry, IPB University (Bogor Agricultural University), Bogor, 16680, Indonesia

³ Department of Animal Health and Veterinary Medicine, Faculty of Veterinary, IPB University (Bogor Agricultural University), Bogor, 16680, Indonesia

⁴ Department of Pharmacognosy and Phytochemical, Faculty of Pharmacy, Airlangga University, Surabaya, 60115, Indonesia

† Corresponding author: Rita Kartika SARI (e-mail: rita_kbu@yahoo.com, ORCID: 0000-0001-5377-1384)

still a health problem in Indonesia. About 4.9 million of the total 262 million people in Indonesia (2%) live in high endemic areas. About 82% of malaria cases in Indonesia found in Papua, West Papua, East Nusa Tenggara (NTT), Maluku, and North Maluku with Annual Parasite Incidence (API) per 1000 inhabitants was 31.93, 31.29, 7.04, 5.81, and 2.77, respectively (Kemenkes, 2017). One effort that can be done in controlling malaria is the distribution of antimalarial drugs. However, commercial antimalarial drugs were derived from synthetic ingredients that have side effects and resistant to chloroquine. The chloroquine substitutes such as mefloquine, halofantrin, and artesunate also appear to have adverse side effects such as mild headaches, itching, lethargy, blurred vision, gastrointestinal disorders, and anorexia that limited the application (Mustofa, 2009). To prevent side effects and resistance, malaria treatment in Indonesia uses a combination of antimalarial drugs, Artemisinin-based-Combination Therapy (ACT) (Depkes, 2012). One of the antimalarial compounds that can be used as an artemisinin combination is plant extractive substances. The combination increases the effectiveness of natural extracts/active compounds and slows down the occurrence of parasitic resistance (Syafii *et al.*, 2016a).

S. ligustrina wood extract is potential to develop as antimalaria drugs. Huda (2006) reported that aqueous extract of *S. ligustrina* wood had an antimalarial activity base on *in vivo* test with mice and suspected that the responsible compounds were flavonoids and alkaloids. Syafii *et al.*, (2016a) reported that the ethanol extracts derived from multilevel extracts of n-hexane and acetyl acetate of *S. ligustrina* wood had a very active antimalarial inhibition for *Plasmodium falciparum* growth. Syafii *et al.*, (2016b) reported the fractions 3 and 4 were classified as very active. The GCMS analysis showed that fractions 3 and 4 of the vaccine were dominated by alkaloid compounds, namely strychnine

and brucine. The results of antimalarial active compounds identification in *S. ligustrina* wood can be used as a standard for standardized antimalarial drug products.

The ethanol extract from previous research using multilevel extraction with n-hexane and acetyl acetate was not recommended. To be categorized as a standardized herbal medicine in Indonesia, the plant extract is only allowed to extract using ethanol and aqueous (BPOM, 2004). Therefore, research on the antimalarial activity of *S. ligustrina* wood extract using ethanol and aqueous and a mixture of both at various concentrations is crucial to produce a safe and active standardized extract. This study aimed to determine the yield extract from *S. ligustrina* wood extract using 100% ethanol (E100), 75% ethanol (E75), 50% ethanol (E50), 25% ethanol (E25), and aqueous (A100); determine the *in vitro* antimalarial activity based on the inhibition of *P. falciparum*; and analyze phytochemical extracts qualitatively and quantitatively with Liquid Chromatography-tandem Mass Spectroscopy (LCMS/MS).

2. MATERIALS and METHODS

2.1. Preparation of Raw Materials

The *S. ligustrina* woods with a diameter of 7-10 cm were collected from NTB, Indonesia. The solvents used for extraction were ethanol and aqueous. Other materials were the Rosewell Park Memorial Institute (RPMI) 1640 containing L-glutamine, gentamicin sulfate injection acid, 0.9% NaCl and 3.5%; N-2-hydroxyl ethyl piperazine-N-2-ethane sulfonic acid (HEPES); gentamicin sulfate; serum and red blood cells (RBC); and citric phosphate dextrose anticoagulant (CPD). The tools used were extractor jars, rotavators, ovens, incubators, Millipore membranes, centrifuge tubes, UV lamps, Laminar Air Flow chambers, desiccators, vortex devices, and light microscopes.

2.2. Extraction

200 g of *S. ligustrina* wood powder sized 40-60 mesh was extracted by maceration method using 100% ethanol, 75% ethanol, 50% ethanol, 25% ethanol, and 100% aqueous. Extraction was repeated 5 times until a clear filtrate was obtained. The filtrate was concentrated with a rotavator to produce 100 mL extract solution. About 10 mL of extract was weighed and put in the oven at 103 ± 2 °C (24 h) for determination of the concentration. The extract weight was obtained by multiply the extract concentration with the extract volume (100 mL). The yield was calculated based on the weight ratio of the extract with powder weight. The yield of each extract was compared using Duncan's test for further analysis. The remaining extract solution (90 mL) was dried in a vacuum oven. The dry extract was used for *in vitro* antimalarial activity test and phytochemical analysis.

2.3. *In vitro* antimalarial activity test

In vitro antimalarial activity testing referred to Trager and Jensen (1976). The stages were incomplete medium preparation, serum preparation, complete medium preparation, erythrocytes preparation, *P. falciparum* parasites breeding, maintenance of *P. falciparum* parasites, synchronization, *in vitro* antimalarial activity in various concentrations (0.01, 0.1, 1, 10, and 100 µg/mL), harvesting, and data analysis. Data analysis included the calculation of the percentage barriers to the growth of *P. falciparum* under a microscope using the formula:

$$\% \text{ Inhibition} = 100\% - [(Nt / Nc) \times 100\%]$$

Information:

Nt = number of live schizonts per 200 asexual *P. falciparum* in testing wells

Nc = number of live schizonts per 200 asexual *P. falciparum* in control wells

Antimalarial activity of each extracts was indicated by the value of 50% Inhibitor Concentration (IC₅₀) in units of µg/mL (Kusch *et al.*, 2011). The IC₅₀ value was determined using a regression equation with a logarithm percent inhibition. The smaller of the IC₅₀ value indicate the higher level of antimalarial activity. The results were classified based on Chinchilla *et al.*, (2011), namely very active IC₅₀ <5 µg/mL, active IC₅₀ 5-50 µg/mL, low IC₅₀ 50-100 µg/mL, and IC₅₀ inactivity > 100 µg/mL.

2.4. Phytochemical qualitative analysis and quantitative analysis with LCMS/MS

Phytochemical analysis was carried out qualitatively and quantitatively. The qualitative phytochemical analysis referred to Harbone (1996). The detected group compounds were alkaloids, flavonoids, saponins, tannins, triterpenoids, and steroids.

2.4.1. Alkaloids

Approximately 3 ml of extracts were added to 3 ml of 1% HCl and heated for 20 min. The mixtures were then cooled and used to perform the following tests:

Dragendoff's test: To crude extracts, 1 ml of Dragendoff's reagent was added drop by drop. The formation of a reddish-brown precipitate indicated the presence of alkaloids.

2.4.2. Phenols

Two milliliters of 5% solution of FeCl₃ were added to 1 ml crude extracts. A black or blue-green colour indicated the presence of phenols.

2.4.3. Tannins

Two milliliters of 5% solution of FeCl₃ were added

to 1 ml crude extracts. A black or blue-green colour indicated the presence of tannins.

2.4.4. Flavonoid

Three milliliters of crude extract was treated with 1 ml of 10% NaOH solution. The formation of an intense yellow colour was an indication of the presence of flavonoids.

2.4.5. Saponins

About 3 ml of crude extracts were added to 3 ml of distilled water and shaken vigorously. The formation of a stable persistent froth was taken as a positive test for saponins.

2.4.6. Steroids

Five milliliters of chloroform and 5 ml of H₂O₄ were added to 500 µl of the prepared crude extracts. The presence of steroids was indicated by a colour change from violet to blue or green or a ring of blue/green or if the upper layer turns red and the sulphuric layer was yellow with a green fluorescence.

2.4.7. Terpenoids

Approximately 2 ml of chloroform and 3 ml of H₂O₄ were added to 5 ml of crude extracts. A reddish-brown coloration was taken as positive test for terpenoids.

The chemical component analysis was carried out on five extracts with a comparison of different ethanol concentrations.

The quantitative analysis used LCMS/MS instruments. In Analyzing mass spectrometers in LC-MS using Shimadzu LC-IT-TOF equipped with ESI. The ESI parameter has a voltage of 4.5 kV, with 200 °C capillary temperature, gas nebulizer 1.5 l / minute. Operation of a mass spectrometer uses positive ion scanning modes from 200 - 2000 m / z. Utilize the Atrantis T3 water column (2.1 - 150 mm) and tem-

perature starting at 40 °C . Binary effluent of (A) 5 mM (NH₄) OAc and (B) CH₃CN solutions in the following gradient conditions: 0-30 minutes linear gradient from 10% - 100% B, 30-40 isocratic minutes at 100% B is the mobile phase. The flow rate is 0.2 ml / minute. The mass spectrometer in MS / MS analysis is in the range of 100-1000 m / z to survey ions and the number of ions entered in the scan to MS / MS analysis automatically until results are obtained. The readable results on the computer are then processed with the *MassLynx* software and adjusted to the available literature.

3. RESULTS and DISCUSSION

3.1 Extract yield

The extract yields of the E100, E75, E50, E25, and A100 from the extraction of *S. ligustrina* wood were varying, which were 3.62-5.30%. The statistical analysis showed that the solvent type affected the yield. Duncan's further test showed that the yield of the E50 was not significantly different from E75, but both were significantly different and higher than other extracts (Table 1). Just like the yield of licorice extract with ethanol solvent, the extract with ethanol concentration was 50% higher than the other concentrations (Ham and Kim, 2019). Solvents with different polarity affect the yield because different solvents dissolve different compounds according to their polarity and availability in extracted materials (Salamah *et al.*, 2008).

The obtained of the E100 extract of *S. ligustrina* wood from this study (Table 1) was higher than the E100 obtained by Syafii (2016a), which was 4.11%. It is due to the application of different extraction techniques. Syafii (2016a) used the ethanol extract obtained from extraction using multilevel polarity solvents (after extraction with n-hexane and acetyl acetate respectively). Hence the non-polar and semi-

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Table 1. The yield of *S. ligustrina* wood extracted by maceration method

Type of extract	Yield (%) ^{*a}
E100	4.32±0.07b
E75	5.19±0.11d
E50	5.30±0.04d
E25	4.70±0.41c
A100	3.62±0.15a

* average of 3 replications,

^a The numbers in the same column followed by the same letters are not significantly different from the Duncan test.

polar extractive substances which can soluble in ethanol are extracted first into the n-hexane and ethyl acetate solvents which are used previously (Houghton and Raman, 1998). In this study, the ethanol extract was obtained from direct extraction using only ethanol solvents. The ethanol solvent is a universal solvent which can attract compounds which are soluble in non-polar and polar solvents and it have an polarity index of 5.2 (Snyder, 1997).

3.2 Antimalarial activity

The *in vitro* antimalarial activity of *S. ligustrina* wood extract showed that all extracts could inhibit *P. falciparum* parasite growth with varying inhibition percentages. Fig. 1 shows for each types of extract, an increase in the extract concentration has increased the inhibition percentage of *P. falciparum* parasites. However, the different types of extracts (based on differences in solvent) at the same of extract concentration caused differences in the inhibition percentage of these parasites. This difference is caused by the type and composition of different extractive substances (Syafii *et al.*, 2016a).

The interpolation of the relationship between the concentration of extract and the inhibition percentage of parasites *P. falciparum* resulted in a regression equation. Based on the regression equation, IC₅₀ value

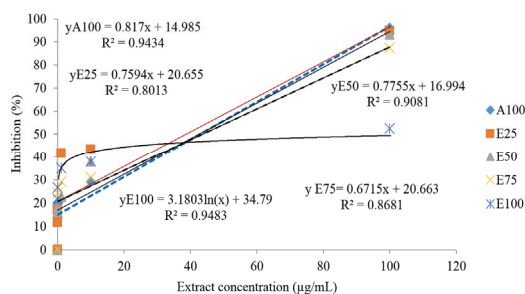


Fig. 1. The relationship Graph of the concentrations of the A100, E25, E50, E75, and E100 extracts of *S. ligustrina* wood with percent of the *P. falciparum* parasite inhibitors and regression equation.

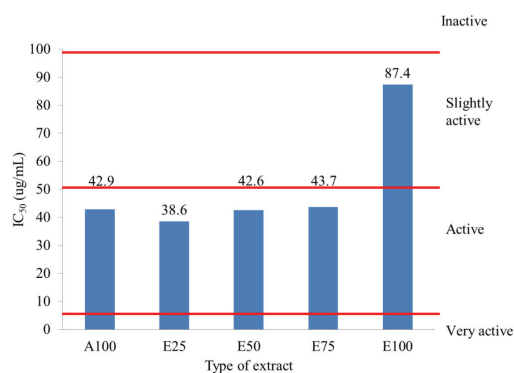


Fig. 2. The IC₅₀ value of the A100, E25, E50, E75, and E100 extracts of *S. ligustrina* wood.

were obtained between 38.6-87.4 µg/mL (Fig. 2). Based on the classification of antimalarial activity which refers to Chinchilla *et al.*, (2011), the A100, E25, E50, and E75 extracts of *S. ligustrina* wood were classified as active because the IC₅₀ values were 5-50 µg/mL. The high antimalarial bioactivity of the ethanol extract is caused by the presence of active compounds from groups of alkaloid compounds, flavonoids, triterpenoids, tannins and hydroquinones found in *S. ligustrina* wood (Syafii *et al.*, 2016a). Ethanol is able to dissolve alkaloids, flavonoids, tannins (Harborne, 1987, Houghton and Raman, 1998), and hydroquinone (Depkes, 1995). Just like extract *Cryptomeria japonica* leaves with acetone and water soluble fractions of methanol extract

and ethanol fraction of hot water extract contained a lot of phenolic compounds and hydroxyl group, indicating that many hydroxyl groups and glycosidic bonds showed higher antioxidant effect than the other fractions (essential oil) (Kim *et al.*, 2017). The hot water extracts of *Ouercus mongolica* and *Rosa rugosa* have potential antioxidant effects (Jung *et al.*, 2017, Kim *et al.*, 2018).

In Syafii's study (2016a), the E100 extract which was classified as very active (IC₅₀ 3.09 µg/mL). It had higher antimalarial activity compared to this study because its IC₅₀ value was lower (Fig. 2). It might be due to the fact that E100 from Syafii's research (2016a) was extracted with multilevel extraction using n-hexane and ethyl acetate hence nonpolar and semi-polar compounds have been extracted first in n-hexane and ethyl acetate. It was confirmed by n-hexane and ethyl acetate extract which were classified as an inactive antimalarial. But in this study, the antimalarial activity of E100 extract was classified as slightly active (Fig. 2). It may occur because ethanol was able to dissolve non-polar and semi-polar chemical compounds contained in *S.ligustrina* wood which resulted in slightly active antimalarial. Ethanol solvents can dissolve both semi-polar and non-polar extractives such as fats, waxes, and other compounds that do not have antimalarial activity (Syafii *et al.*, 2016b).

3.3 Phytochemical profile

The qualitative phytochemical analysis showed that *S. ligustrina* wood extract was detected to contain groups of extractive substances in various intensities. It is because different types and polarity of solvents affect the type and composition of extracted phytochemical compounds. In addition, the solvent type will also affect the extracted bioactive compounds (Tiwari *et al.*, 2011). In Table 2, the A100 and E25 extracts that have antimalarial activities which are classified as active (Fig. 2) were detected to contain very strong and strong flavonoids (Table 2). According to Widyawaruyanti *et al.* (2011), the group of flavonoids, especially prenylated flavones and chalcones, are reported to have high antimalarial activity. According to Bilia *et al.* (2006), the flavonoid group has an action mechanism by inhibiting the nutrients transport and inhibiting the degradation and detoxification of hemoglobin in *Plasmodium*. The E50 and E75 extracts were also classified as having antimalarial activities were detected to contain low flavonoids. But it detected to contain strong and very strong alkaloid, respectively. The alkaloids like strychnine had strong antimalarial activity (Syafii *et al.*, 2016b). However, there has been no report about brucine antimalarial activity. Succeeded in isolating brucine in *Strychnos nuxvomica* plant which has anti-immfamatory and analgesic activity (Yin *et*

Table 2. Results of qualitative phytochemical analysis of *S. ligustrina* wood extract

Active compounds	The intensity of detection in qualitative analysis					
	E100	E75	E50	E25	A100	Powder
Alkaloids	+++	++++	+++	+++	++	++
Phenol	+++	++	++	++	+	+++
Hydroquinon						
Tannins	+	+	+	++	+	-
Flavonoids	+	+	+	+++	++++	++
Saponins	+	-	-	++	+	++++
Steroids	+	+	+	+	+	+
Terpens	+	+	++	++	++	++

- : undetectable, +: weak, ++: medium, +++: strong, ++++: very strong

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al., 2003). But brucine is a compound with the third largest relative concentration from *S. ligustrina* fraction 4 (very active antimalarial IC₅₀ 0.386 µg/ml) (Syafii *et al.*, 2016b).

All extracts were containing various concentration of alkaloid compounds. Alkaloids were identified from the amount of sediment obtained by the addition of dragendorff reagents. Larger sediment means higher alkaloid content. The E75 extract was detected to contain very strong alkaloids, while the E100, E50, and E25 extracts were strong and the A100 extract was moderate (Table 2). *S. ligustrina* wood contains four alkaloid compounds, namely colobrin N-oxide, strychnine Noxide, brucine, and brucine N-oxide. The higher alkaloids content are found in the stem and root of the plant (Hadi and Bremner, 2001). The action mechanism of alkaloids as an antimalarial is through inhibition of haem parasitic detoxification in food vacuoles, especially potential as blood schizonticide and gametocide (Hadi and Bremner, 2001, Depkes, 2008, and Mustofa, 2009).

The phytochemical test conducted by Frederich *et*

al. (1999) revealed that the wood of the genus *Strychnos* contain strong alkaloid compounds and have the potential to be antimalarial. Sadono's research (2011) of the methanol extract of *S. ligustrina* wood has four main components, one of which is Strychnine which is one of the antimalarial compounds. The detection of alkaloid and flavonoid compounds in the *S. ligustrina* wood extract in this study is in line with previous studies. Syafii (2016a) showed that the E100 extract was detected to be very strong in alkaloids and strongly contain flavonoids and phenol hydroquinone. Therefore, the examination of *S. ligustrina* wood is crucial.

The LCMS/MS chromatograms of the E75, E50, and E25 extracts had similar patterns. The metabolite profiling using LCMS/MS produced three complex data matrices which include the value of the mass to charge ratio (m/z), retention time, and peak intensity. The five extracts were identified to contain furan compounds, phenolic, aldehydes, alkaloids, fatty acids, lignans, and quinones with varying relative concentrations (Table 3). Table 3 shows that the main compounds of the five types of extract was brucine with different relative

Table 3. The results of LCMS / MS analysis of *S. ligustrina* wood extract

Group of compounds	Compound	Formula	m/z (g/mol)	Retention time (minutes)	Relative concentration (%)				
					E100	E75	E50	E25	A100
Aldehyde	5-hidroksimetilfurfural	C ₆ H ₆ O ₃	126.111	2.05	-	0.001	0.01	0.02	0.02
	Guaiacol	C ₇ H ₈ O ₂	124.139	2.05	0.12	0.04	0.54	0.02	3.54
	Vanillin	C ₈ H ₈ O ₃	152.149	1.47	0.01	0.001	0.01	0.01	4.68
Alkaloids	Strychnine	C ₂₁ H ₂₂ N ₂ O ₂	334.419	3.90	0.12	0.04	0.54	0.94	3.54
	Brucine	C ₂₃ H ₂₆ N ₂ O ₄	394.471	5.61	11.62	11.79	21.33	24.55	24.96
Fatty acid	Palmitic acid	C ₁₆ H ₃₂ O ₂	256.43	4.77	0.44	0.49	0.97	1.16	3.54
	9,11-octadecadienoic acid	C ₁₈ H ₃₂ O ₂	280.452	2.05	-	0.01	0.01	0.02	0.02
	Phyllamyricin A	C ₂₂ H ₁₈ O ₇	394.380	4.77	0.44	0.49	0.97	1.16	4.68
Lignans	2-metoksihidrokuinon	C ₇ H ₈ O ₃	140.138	0.39	-	-	0.001	0.01	0.001
Quinones	Antrasena	C ₁₄ H ₁₀ (C ₆ H ₄ CH) ₂	178.234	0.39	-	-	0.001	0.01	0.001
	9,10-bis(4-metoksifenil) antrasena	C ₂₈ H ₂₂ O ₂	390.482	8.09	0.78	0.29	0.98	0.62	2.49

concentrations. The E25 extract as the most active extract (IC₅₀ value 38.6 µg/mL) contained brucine with a relative concentration of 24.55%, while extract of the E100 extract as an extract with the lowest antimalarial activity (IC₅₀ value 87.4 µg/mL) contained brucine with the lowest concentration of 11.62%. Although none of the literature reviews has reported brusin as an antimalarial compound, Syafii (2016b) reported that fraction four as the most active antimalarial fraction in the ethanol extract of *S. ligustrina* wood contained brucine. Another compound that plays a role in antimalarial activity is strychnine. Strychnine is an alkaloid compound found in parts of *S. ligustrina* wood (Setiawan *et al.*, 2014) and has antimalarial activity (Frederich *et al.*, 1999). In this study, the E25 extract contained 0.94% of strychnine, while the E100 extracts contained the lowest, which was 0.12% (Table 3).

The A100 extract contained higher brucine and strychnine compared to the E25 extract (Table 3). However, the IC₅₀ value of the A100 extract was slightly lower than the E25 extract (Fig. 2). The A100 extract had a lower IC₅₀. It may be caused by the high concentration of non-antimalaria compounds such as palmitic acid; 9.11 octadecadienoic acid; Guaiacol; Phyllamycin A; and 9, 10-bis (4-methoxyphenyl) anthracene. The literature review showed that these compounds have been no reports on antimalarial activity (Syafii *et al.*, 2016b).

4. CONCLUSION

The yield of *S. ligustrina* wood with ethanol and aqueous solvents at various concentrations was varied. The highest yield was obtained in the E50 extract (5.30%), followed by E75 (5.19%), E25 (4.70%), E100 (4.32%), and A100 (3.62%). The *S. ligustrina* wood extracts of E25, E50, A100, and E75 had an active antimalarial activity with IC₅₀ value of 38.6 µg/mL, 42.6 µg/mL, 42.9 µg/mL, and 43.7 µg/mL, respectively.

However, the E100 extract was classified as slightly active with IC₅₀ value of 87.4 µg/mL. The qualitative phytochemical analysis showed that all extracts detected to contain strong alkaloids and phenolic compounds. The LCMS/MS analysis showed that the main compounds of all extracts were brucine and strychnine.

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