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Ultrasound-Assisted Extraction of Antimalarial Compounds from the Bidara Laut (*Strychnos ligustrina*) Heartwood

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ABSTRACT

The aqueous wood extract of bidara laut (*Strychnos ligustrina*) from maceration demonstrated the greatest antimalarial activity against *Plasmodium falciparum* strain 3D7. However, this method has several limitations, including a lengthy extraction time, which allows for microorganism contamination. The aim of this study was to quantify the yield, evaluate the antimalarial effectiveness of the *P. falciparum* growth in-vitro, and analyze the aqueous bidara laut heartwood extracts phytochemical composition from the two different extraction methods, namely the method of maceration extraction for 24 hours and the ultrasound-assisted extraction (UAE) for 30 (UAE30), 45 (UAE45), and 60 (UAE60) minutes. The bidara laut heartwood extract from the UAE60 method had relatively similar yield and antimalarial activity with the bidara laut heartwood extract from the UAE45 method. However, these extracts had higher yield and antimalarial activity than the extracts from the maceration and the UAE30 methods. The results of LC-MS/MS analysis revealed that the main compounds in the UAE45 and UAE60 extracts were strychnine, quinaprilat, and 3,4-bis-cyclobut-3-ene-1,2-dione, which has potent antimalarial properties.

1. Introduction

The *Plasmodium falciparum* parasite causes malaria, transmitted through the bite of an infected mosquito. The World Malaria Report in 2021 reported that there are 240 million cases of malaria, which cause 600,000 deaths worldwide (Dvorin and Goldberg 2022). In Indonesia, a total of 250,644 cases of malaria have been identified (Hussein et al. 2020). A new approach has been devised by the Indonesian Ministry of Health for treating malaria by combining artemisinin and natural antimalarial bioactive compounds (Ministry of Health RI 2019). Bidara laut (*S. ligustrina*) wood extract can be a source of antimalarial substances. Empirically, the people in West Nusa Tenggara and Bali use it as an antimalarial herb (Setiawan and Narendra 2012). Bidara laut trees are widely distributed in Bali and West Nusa Tenggara, especially in Bima and Dompu Regencies (Setiawan et al. 2014). Bidara laut wood is scientifically proven to contain antimalarial phytochemicals (Manurung et al. 2019; Syafii et al. 2016, 2017). The in vivo test results showed

that the rats given the water-soluble bidara laut wood extract was able to inhibit the *P. berghei*'s growth by 74%, while a combination of this extract with dihydroartemisinin and piperaquine phosphate (DHP) was able to inhibit the *P. berghei*'s growth by 94%. It exhibits significant antimalarial and antipyretic effects (Cahyaningsih et al. 2022).

In previous investigations, the type of extraction solvents used has been demonstrated to influence the bidara laut wood extract yield and antimalarial activity (Syafii et al. 2017). The bidara laut wood extract from maceration with 100% ethanol had higher yield and antimalarial activity than those from maceration with ethyl acetate and n-hexane (Syafii et al. 2017). The antimalarial activity of the fourth fraction from fractionation of this bidara laut wood extract is rated very active (Syafii et al. 2016). Manurung et al. (2019) reported that bidara laut wood extract from maceration with water had higher antimalarial activity than from maceration with 25-100% ethanol. It demonstrated that the maceration of bidara laut with water produces the extract with the best antimalarial activity and is relatively inexpensive. However, maceration with water has disadvantages, such as a long extraction period that allows microorganisms to grow (Suryani et al. 2016). For this reason, a quicker extraction method than maceration is required.

Ultrasound-assisted extraction (UAE) is quicker than the maceration method (Altemimi et al. 2017). Priscilia et al. (2020) reported that the UAE extract (12.39%) of *Kjellbergiodendron celebicum* produced a higher yield than the extract from maceration (9.25%) with the same extraction time of 24 hours (IC50 UAE is 9.81512 µg/mL) and maceration of 11.48336 µg/mL. Saini et al. (2019) also showed that the maceration extract of peels of *Citrus reticulata* and *Citrus limetta* for 24 h produced a yield of 5.20% and a DPPH scavenging activity value of 22.46%, while extracts from the UAE for 30 minutes produced a yield of 12.95% with a DPPH scavenging activity value of 48.23% (Saini et al. 2019). The UAE employs ultrasonic waves, which cause damage to the thin layer of cell walls and help retain phenolic compounds trapped in the cell walls (Handaratri and Yuniati 2019), thereby reducing extraction time (Wen et al. 2018). Other advantages of the UAE extraction method are that it can increase the yield, quality, and functionality of extracts (Li et al. 2021; Zhao et al. 202), is effective for thermolabile materials, is environmentally friendly (Zhang et al. 2018), and can extract the extracts from the matrix without destroying the structure of the extract (Babaei et al. 2006). The UAE extraction of mangosteen peels with ethanol 95% for 45 minutes can increase the yield by 6.71% with very high antioxidant activity (IC_{50} 4.93 ppm) (Sholihah 2017). Meanwhile, the results by Buanasari et al. (2019) stated that an extraction time improved by 30 minutes at 200 W of power and a frequency of 14 kHz. Budiastra et al. (2021) confirmed that extraction times of 30, 45, 60, and 75 minutes increased the yield of nutmeg oleoresin by 11 to 52%. This result shows that the longer extraction time of the UAE method produces extracts with higher yields. However, UAE also has the disadvantage that too long extraction times will result in more actual energy use and may result in increased extraction costs. Therefore, this study aims to quantify the yield, test the in vitro antimalarial, and analyze the phytochemicals of the bidara laut wood extracts from the maceration method for 24 hours and the UAE method at different extraction times.

2. Materials and Methods

2.1. Materials

A total of 3 bidara laut trees with diameters of 14.5 cm, 15 cm, and 17 cm were taken from Dompu District, West Nusa Tenggara. The part of the stem used was the heartwood stem. Wood

samples were made into 40-60 mesh powders using a Wiley mill grinder (LabForce®, Thomas Scientific, US). Before extraction, the samples were measured for water content by taking ± 2 g samples and up until they attained a consistent furnace dry weight at 103 ± 2°C.

2.2. Methods

2.2.1. Maceration extraction method

As much as 150 g of bidara laut powder was extracted by maceration method using water solvent (Manurung et al. 2019). Extraction was carried out with a powder and a 1:10 (w/v) solvent ratio at room temperature. Extraction was carried out for 24 hours, then filtered, and re-extraction was carried out on the same residue with three times a new solvent. The extraction procedure was done in a room temperature. The filtrate obtained was vaporized under vacuum at 50°C to produce a dry extract. The yield was then determined by weighing the dry extract, tested for antimalarial activity, and analyzed for its phytochemical profile. The repetition extraction process was repeated three times.

2.2.2. Ultrasound-assisted extraction (UAE) extraction method

As much as 150 g of dry powder from bidara laut heartwood was extracted by the UAE method using a water solvent with a ratio of materials and solvents of 1:10 (w/v). The powders and solvents were subjected to ultrasonic waves using an ultrasonic bath (Powersonic 405, Hwansin Technology Company, Korea) with variations in extraction time of 30, 45, and 60 minutes (Budiastra et al. 2021). Ultrasonication was applied at 65% amplitude and 40 kHz frequency. Re-extraction of the same residue with three times a new solvent. The obtained solution was filtered. The filtrate obtained was evaporated under a vacuum at 50°C to produce a dry extract, and its yield was calculated. Then the antimalarial activity was tested, and the phytochemical profile was analyzed. The repetition extraction process was repeated three times.

2.2.3. Phytochemical analysis using LC-MS/MS

The instrument used was UHPLC (ThermoScientific, US) with AccucoreTM column C18 100 × 2.1 mm, 1.5 m. As much as 0.5 µL of the filtrate was injected into the UHPLC-Q-Orbitrap-MS/MS (Thermo Fisher Scientific, Waltham, MA, USA) after the metabolite was separated using a gradient elution system for 50 minutes at a flow rate of 0.2 mL/minute. The composition of the mobile phase used was 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B), with a gradient elution system for 0-1 minute (5% B), 1-25 minutes (5-95% B), 25-28 minutes (95% B), 28-30 minutes (5% B). Positive Electrospray Ionization (ESI) was used, with Q-Orbitrap serving as the mass spectrometer in the electrospray ionization mode. The m/z range was 100-1500. The capillary temperature is 320°C, the column temperature is 30°C, the spray voltage (+) 3.8 kV, the sheath flow rate is 15 mL/minute, and the auxiliary gas flow rate is 3 mL/minute. The resolution utilized is 70,000 FWHM. Full MS/dd MS2 scans were employed. Mass spectra were obtained from UHPLC-Q-OrbitrapMS/MS and analyzed with Compound Discoverer version 2.3, MSDial, and MSFinder (Thermo Fisher Scientific, Waltham, MA, USA). The databases used were mzcloud, ChemSpider, HMDB, and MassBank. Compounds with high relative abundance in each type of extract were selected and then summarized.

2.2.4. Antimalaria activity test

In vitro, antimalarial test refers to Manurung et al. (2019). The strain of Plasmodium used was *P. falciparum* 3D7, which is chloroquine-sensitive. The test sample was created by combining 10 mg of the sample with 1000 µl DMSO (stock solution, 10,000 µg/mL in concentration). From the stock dilution, successive dilutions were performed to produce concentrations of 0.01 µg/mL, 0.1 µg/mL, 1 µg/mL, 10 µg/mL, 50 µg/mL, and 100 µg/mL. This test employed synchronous parasites (ring stage) with parasitemia of ±1% (5% hematocrit).

The test procedure was performed by adding up to 2 µL of the test solution to each well (well 96), then adding 198 µl of parasites (multiple concentrations were made). The test well was inserted into the chamber and fed with a gas mixture (5% oxygen, 5% carbon dioxide, and 90% nitrogen). For 48 hours, the chamber was incubated at 37°C. After the cultures were harvested, the 20% Giemsa stain was used to make thin blood films.

Data analysis was performed on blood smears and the number of infected erythrocytes over 1000 normal erythrocytes. The data are then used to determine the growth and percent inhibition. The following equations obtained percentage of parasitemia was by the following equations:

$$\%\ parasitemia = \frac{Infected\ erythrocytes}{1000\ erythrocytes} \times 100\% \quad (1)$$

$$\%\ inhibition = \frac{Parasitemia\ test}{Parasitemia\ control} \times 100\% \quad (2)$$

It is considering the findings on the percentage of inhibition. , statistical analysis was performed using probit analysis through the relationship between the percentage of parasite inhibition and the extract concentration to calculate the IC_{50} value, which stands for the test substance that can inhibit parasite growth by 50%. A lower IC_{50} value indicates the highest antimalarial activity (Turalely et al. 2018).

According to Rasoanaivo et al. (2004), antimalarial effectiveness was classified. The extract is classified as very active if the IC_{50} value is less than 5 µg/m, and classified as active if the IC_{50} value is from 5-50 µg/mL, classified as weakly active if the IC_{50} value is from 50-100 µg/mL, and classified as inactive if the IC_{50} value is more than 100 µg/mL.

2.3. Data Analysis

The yield and IC_{50} values were analyzed using the variance analysis in a Complete Random Design. The treatment applied to this investigation was the difference in the extraction method with the treatment levels of the UAE extraction method for 60 minutes, UAE for 45 minutes, UAE for 30 minutes, and the maceration method. If the variance analysis results show that treatment affects the yield or IC_{50} value, the subsequent data analysis step is Duncan's different test (Hanafiah 2016).

3. Results and Discussion

3.1. Yield

The yields of bidara laut heartwood extracts dissolved in water obtained using the maceration method and the method of UAE at various extraction times were varied (**Table 1**). The analysis of variance revealed that the difference in the extraction method of bidara laut heartwood

significantly affected the extract's yield value ($p < 0.05$). The yield extract from the UAE method for 45 minutes did not differ substantially from the UAE method for 60 minutes. The results of Duncan's multiple range test indicated that the yield extract from the method of UAE for 45 minutes was not significantly different from the yield extract from the method of UAE for 60 minutes. Compared to the UAE method for 30 minutes and the maceration method, the extract yield from the two methods was higher (**Table 1**). The trend was that the longer the UAE extraction time, the higher the extract yield. It was most likely due to the solvent taking longer to penetrate the cell wall and remove the compounds in the material (Isdiyanti et al. 2021). As a result, the levels and composition of compounds successfully extracted increased.

Table 1. Extract yields of bidara laut heartwood based on the type of extraction method

Type of extraction method	Yield (%)
Maceration	6.62 ± 0.54b
UAE 30 minutes	5.82 ± 0.20a
UAE 45 minutes	7.39 ± 0.28c
UAE 60 minutes	7.95 ± 0.28c

Notes: the letter of the value shows a significant difference with a p-value lower than 0.05 with Duncan's multiple-range test.

Compared with yields from macerated extracts, extraction with the UAE method for 45 and 60 minutes resulted in higher yields **(Table 1).** High extract yields in the UAE method can be achieved because ultrasonic waves break the cell walls during the extraction process. Thus the cell components exit and amalgamate with the solution (Budiastra et al. 2021). However, the yield extract from the UAE method at 30 minutes (5.82± 0.20%) was lower than that from maceration. It demonstrated that the solvent in the UAE method does not have enough time to dissolve the compound for 30 minutes.

3.2. Antimalaria Activity

In vitro, antimalarial activity tests revealed that all extracts may inhibit the growth of *P. falciparum* strain 3D7 with various levels of inhibition. **Fig. 1** demonstrates that the percentage of *P. falciparum* inhibition increased with increasing concentrations of bidara laut heartwood extracts. However, the percentage of inhibition varied by the extract. The extract concentrations of 0.01 µg/mL, 0.1 µg/mL, 1 µg/mL, and 10 µg/mL were unable to inhibit parasite growth by 50%. The extract concentrations above 50% inhibition were 100 µg/mL and 50 µg/mL, with the highest inhibition being 100 µg/mL. This was presumably due to the presence of antimalarial compounds in the extract, where higher concentration extracts result in lower parasitemia and increased inhibition of *P. falciparum* growth.

The antimalarial activity of aqueous extracts of bidara laut heartwood from various extraction methods varied based on the IC_{50} value **(Table 2).** The IC_{50} value of the bidara laut heartwood extract using the UAE method ranged from 4.13 ± 0.16 µg/mL to 5.84 ± 0.20 µg/mL, while the macerated extract was 9.65 ± 0.30 µg/mL. Statistical analysis revealed that the various bidara laut heartwood extraction methods significantly inhibited *P. falciparum* growth. Duncan's multiple range test indicated that the extract from the UAE method for 60 minutes (UAE60) had antimalarial effectiveness that was not significantly different from the extract of the UAE method for 45 minutes (UAE45). However, the antimalarial effectiveness of the two extracts was significantly higher and different from the UAE method extract for 30 minutes (UAE30) and the

extract by the maceration method (EM) (**Table 2**). The category of antimalarial activity based on the IC_{50} value refers to the class of an extracted compound in the in vitro test. According to Rasoanaivo et al. (2004) that the antimalarial activity is very good if the IC_{50} value is 1.0–10 µg/mL, the IC_{50} activity is weak > 10–25 µg/mL, the IC_{50} activity is very weak 25–50 µg/mL and is in the inactive category if the IC_{50} value is > 100 µg/mL. This result showed that the UAE30 and EM were classified active as antimalarials, whereas the UAE45 and UAE60 were classified as very active.

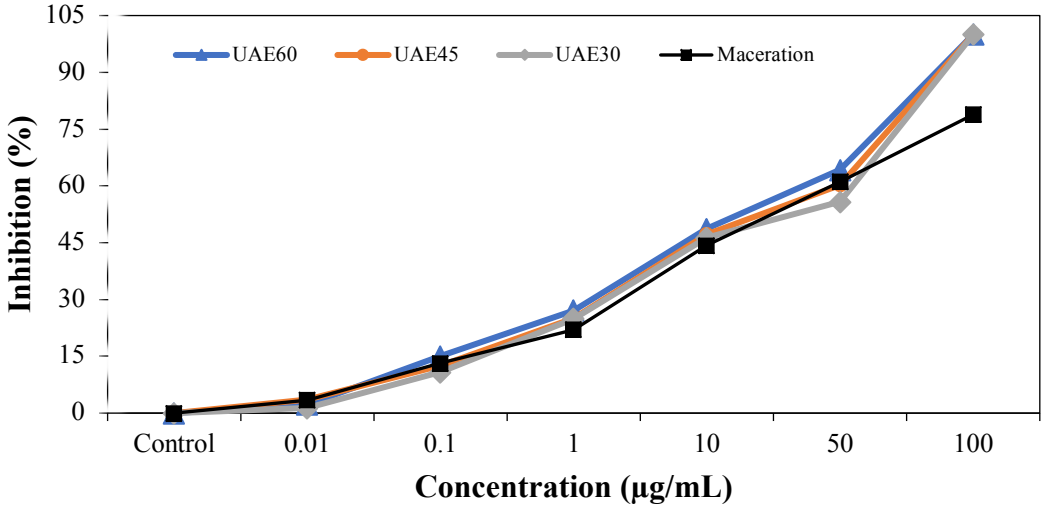

Fig. 1. The relationship between the bidara laut extract concentrations from the UAE method with percent inhibition *P. falciparum.*

Table 2. The IC_{50} value of bidara laut heartwood extracts based on the type of extraction method

Type of extraction methods	IC_{50} (µg/mL)
Maceration	9.65 ± 0.30c
UAE 30 minutes	5.84 ± 0.20b
UAE 45 minutes	4.91 ± 0.27a
UAE 60 minutes	4.13 ± 0.16a

Notes: the letter of the value shows a significant difference with a p-value lower than 0.05 with Duncan's multiple-range test.

The antimalarial activity of the bidara laut heartwood extract resulting from the UAE method was higher than that of the maceration method due to the IC_{50} value. However, the UAE30 extract yield was lower **(Table 2**). This result was presumably because the UAE method's ultrasonic waves can produce extracts with higher antimalarial activity than the maceration method. The chemical compounds in the macerated extract have lower solubility than the extract produced using ultrasonic waves (Babaei et al. 2006).

3.3. Phytochemical Profile

The LC-MS/MS analysis revealed that the water-dissolved bidara laut heartwood extract's phytochemical content from the maceration and UAE methods varied with extraction time. There were 51 compounds with varying relative concentrations. At least 12 compounds have a similarity index greater than 90% (**Table 3**).

Alkaloid compounds dominated all extracts. Syafii et al. (2016) discovered that alkaloid compounds derived from the ethanol extract of bidara laut wood are effective antimalarials. Although the yield of the UAE extract for 30 minutes was lower than 45 and 60 minutes, based on

the results of this LCMS study, extracts from the UAE technique showed the highest antimalarial activity for 30 minutes, which contained several compounds that were active as antimalarials, namely quinaprilat, 3,4-bis-cyclobut-3-ene-1,2-dione, pravadoline, and N6-threonylcarbamoyladenosine. Strychnine, quinaprilat, and 3,4-bis-cyclobut-3-ene-1,2-dione were the alkaloid compounds that dominated these extracts. Manurung et al. (2019) found the strychnine compound to be antimalarial. Matthews (2015) stated that quinaprilat is the most effective compound inhibiting *P. falciparum*. Lande et al. (2021) also discovered that the compound 3,4-bis-cyclobut-3-ene-1,2-dione inhibit protein kinase plasmodial, which had a significant impact on the parasite malaria's life cycle (Arendse et al. 2021). The findings of this study demonstrated that aqueous bidara laut heartwood extract from the maceration and UAE extraction methods could dissolve antimalarial alkaloid compounds. Wardani et al. (2021) stated that an extract with an IC_{50} value of less than 5 µg/mL could be declared to have very active antimalarial activity.

Table 3. LC-MS/MS analysis result of bidara laut heartwood extracts

No.	Retention time	Metabolic name	MW	Relative abundance (%) in extracts			
				EM	UAE 30	UAE 45	UAE 60
1	7.70	*(-)-Strychnine*	334.16	15.35	15.05	15.14	16.05
2	8.04	*Quinaprilat*	324.18	12.65	12.98	13.39	13.59
3	7.35	*3,4-bis-cyclobut-3-ene-1,2-dione*	348.18	10.01	10.60	11.53	11.57
4	1.33	*D-(+)-Proline*	115.06	8.48	8.73	9.22	9.96
5	1.24	*Choline*	103.09	12.74	8.92	9.64	9.71
6	7.35	*4-methoxyphenyl*	390.05	5.41	2.07	3.11	4.54
7	7.76	*Pravadoline*	378.19	2.51	3.24	3.60	3.54
8	7.89	*(E)-4-Methoxycinnamic acid*	178.18	2.40	1.81	1.88	1.93
9	7.44	*Loganin*	390.15	2.70	2.82	2.79	3.25
10	10.00	*Cinchonine*	516.12	0.81	0.73	0.77	1.12
11	1.36	*Cartnitine furmate*	277.11	4.66	1.69	2.01	2.95
12	7.44	*N6-threonylcarbamoyladenosine*	412.13	2.28	2.72	2.97	3.11
13	5.98	*Koumine*	306.17	0.06	0.02	0.02	0.02
14	7.57	*Quinine*	410.18	0.01	0.01	0.01	0.01

The LC-MS/MS analysis demonstrated that the highest relative concentrations of strychnine, quinaprilat, and 3,4-bis-cyclobut-3-ene-1,2-dione were found in the UAE60 extract. Other extracts contained these three compounds in lower concentrations (**Table 3**). When the relative concentrations of the three compounds were compared to the IC_{50} value (**Table 2**), there was a tendency to decrease IC_{50} with the increase in the relative abundance of the three compounds in the extract (**Table 3**). The lower IC_{50} value reflected the higher antimalarial activity (**Table 2**). Strychnine, quinaprilat, and 3,4-bis-cyclobut-3-ene-1,2-dione compounds in UAE extract increased its antimalarial activity over macerated extract. These findings demonstrated that the UAE method was more effective than maceration because the UAE method extracted antimalarial compounds faster.

The UAE30 extract contains an antimalarial compound, namely strychnine, with a higher concentration than the EM extract (**Table 3**). However, the antimalarial activity of the UAE30 extract was higher than that of the EM extract. This is presumably because the UAE30 extract contains several compounds that are also antimalarial at higher concentrations than extracts from EM, namely quinaprilat, 3,4-bis-cyclobut-3-ene-1,2-dione, pravadoline, and N6-threonylcarbamoyladenosine. In addition, several non-antimalarial compounds such as D-(+)-

proline, choline, (E)-4-methoxycinnamic acid, and 9,10-bis(4-methoxyphenyl) anthracene are classified as fatty acids with relatively the highest concentrations in macerated extracts. D-(+)-proline is an antioxidant (Altaf et al. 2022), choline is an anti-inflammatory (Kusuda et al. 2020), (E)-4-Methoxycinnamic acid and 9,10-Bis(4-methoxyphenyl) anthracene has antibacterial and antioxidant activity (Syafii et al. 2016). So it is suspected that these compounds affect the activity of antimalarial compounds in EM extracts.

4. Conclusions

In general, the ultrasound-assisted extraction (UAE) method can shorten extraction time without reducing antimalarial activity. The extraction using the method of UAE from the heartwood of bidara laut for 45-60 minutes had higher yields and antimalarial activities than the extracts from the UAE for 30 minutes and the maceration method. The UAE extract for 45 minutes and UAE extract for 60 minutes were classified as very active as antimalarials because these extracts could inhibit the *P. falciparum* growth with IC_{50} values of 4.91 µg/mL and 4.13µg/mL, respectively. The LC-MS/MS analysis revealed that *strychnine*, *quinaprilat*, and *3,4-bis-cyclobut-3-ene-1,2 -dione* was the main UAE45 and UAE60 extracts compounds considered to contribute to antimalarial activity.

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