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RESEARCH ARTICLE

In vitro antimalarial activity assay of Ashitaba Leaf ethanolic extract (*Angelica keiskei*)

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Abstract

Introduction: The incidence of malaria is still very high in number in the world. Difficulty in treating malaria is caused by the resistance of malaria parasites to conventional drugs. An alternative treatment that can be used to treat malaria is to discover new drugs from natural ingredients. **Aim:** This study aimed to determine the activity of the Ashitaba leaf ethanolic extract as an antimalarial drug to *Plasmodium falciparum* strain 3D7. **Methods:** This study tested the activity of Ashitaba extract on the growth of *P.falciparum* in five concentrations, namely concentration of 0.01 ppm, 0.1 ppm, 1 ppm, 10 ppm, and 100 ppm. **Results:** The test results showed that the highest inhibitory effect was found on the concentration of 100 ppm with percent inhibition of 79.47 ± 26.91%. The 50% inhibition to parasites showed the half maximal inhibitory concentration (IC_{50}) value of 2.09 ppm, compared to the positive control of which the IC_{50} of chloroquine was 0.007 ppm. **Conclusion:** Ashitaba leaf extract can be considered to have very active anti-malarial activity, because it has an IC_{50} value of less than 5 ppm.

Introduction

Malaria is a dangerous disease caused by parasites that are transmitted to humans through the bite of a female Anopheles mosquito infected with the parasite (Sudoyo, 2009). In 2018, there were an estimated 228 million cases of malaria worldwide. The estimated number of deaths from malaria was 405,000 in 2018. Children under five years of age are the group most vulnerable to malaria, accounting for 67% of all malaria deaths worldwide. The African region bears the global malaria burden. In 2018, Africa was home to 93% of malaria cases and 94% of malaria deaths (Dinkes N.T.B., 2018).

Indonesia was reported as the third-highest number of malaria cases in the Southeast Asia region, amounting to 229,819 cases. Likewise, the number of deaths was 432 (WHO, 2020). Although the incidence of malaria has tended to decrease since 2000, there are still malaria outbreaks in seven provinces that have attacked 35 villages and caused the death of 211 residents. In Indonesia, the number of malaria sufferers tends to decline from year to year. However, several provinces in Indonesia still suffer from malaria, especially in the eastern part of Indonesia, namely Papua and West Papua, including West Nusa Tenggara (Depkes R.I., 2006).

The development and discovery of antimalarial drugs are expected to provide new drugs with potential and safe drug targets and mechanisms for humans. The emergence of drug-resistant *Plasmodium* species to antimalarials has urged researchers to look for new antimalarials to replace ineffective antimalarials. One of the efforts to find new antimalarials is through research on medicinal plants that are traditionally used by the community to treat malaria (Depkes R.I., 2008).

One of the plants used in an antimalarial treatment is the Ashitaba plant because this plant contains active substances that have medicinal functions. Research at the Osaka Pharmacy University in 1990 showed that the active ingredients contained in 100g of ashitaba were 0.25% xanthoangelol, 4-Hydroxyderricin 0.07% and 0.32% total chalcone. Ashitaba contains hexadecanoic acid 2.42%, palmitic acid 5.08%, xanthotoxin 3.12%, linoleic acid 9.17%, pyrimidine 2.70%, strychnidinone 3.18% and smenochromena 7.55% (Mustofa, 2009). Chalcone (1,3-diphenyl-2propen-1-on) is a compound that contains two aryl rings connected with ketones α, β unsaturated. Chalcone is an important intermediate in organic synthesis. The chalcone group is a structure common in plants that contain secondary metabolites of flavonoids (Handayani *et al.*, 2013). Chalcone has also been reported as a potential antimalarial agent (Hans *et al.*, 2010).

Material and method

Materials

The materials in this research were micropipette, Erlenmeyer flask, measuring cup, glass jar, analytical balance, laminar air flow (LAF), incubator, refrigerator, mix gas, pipette, petri-dish, well 96, sterile medium bottle, centrifuge, microscope, water bath, glass stirrer, oven, silica gel GF254, chamber, microtube, ethanol, and Ashitaba leaves (*Angelica keiskei* K), parasites used are *Plasmodium falciparum* strain 3D7 parasites, 50% dimethyl sulfoxide (DMSO), Roswell Park Memorial Institute (RPMI), 20% Giemsa colouring and 5% sorbitol, chloroquine.

Methods

Extraction of Ashitaba Leaves

Ashitaba leaf ethanolic extracts were made from 100 gram of Ashitaba simplicia leaves immersed in 400 ml of 70% ethanol mixture, stirred every three hours for 15 minutes for 24 hours. The immersion results were squeezed, then left for ten minutes before being evaporated at control temperature (50^{o}C) to obtain a thick extract.

Phytochemical screening

Wilstater test: Two ml of Ashitaba leaf extract solution was added with ten drops of concentrated hydrochloric acid (HCl) and a little magnesium powder. The orange-yellow colour indicates the presence of flavones, chalcone, and auron, based on the results of research by Sofa Fajriah & Megawati (2015).

Bate Smite-Metcalfe test: Two ml of Ashitaba leaves extract solution was added with ten drops of concentrated HCl then heated. The red colour indicates the presence of flavonoids.

Antimalarial activity assay

Sample preparation: One mg sample was dissolved in 100 µl DMSO (stock solution, concentration of 10,000 µg / ml). The serial dilution was made from a stock solution to obtain a final concentration of 1000 ppm, 100 ppm, 10 ppm, and 1 ppm.

Parasite preparation: The parasites used in this test were synchronized ring stage with parasitemia ± 1%.

Procedure: Two µl of the test solution with various concentrations was taken and put in each microwell, then 198 µl of the parasite was added until the test sample had a final concentration of the test sample of 100 ppm, 10 ppm, 1 ppm, 0.1 ppm, and 0.01 ppm. The microwell was then put in a chamber and given a mixed gas (5%, O_2, 5% CO_2 and 90% N_2). The chamber containing was incubated for 48 hours at a temperature of 37^{o}C. The culture was then harvested, and a thin blood smear was made with 20% Giemsa staining.

Statistical analysis

Blood smears that have been made were calculated by counting the number of infected erythrocytes every 1,000 normal erythrocytes under a microscope. The data were then used to determine the per cent growth and per cent inhibition. Per cent growth was obtained by the following formula:

$$\% \text{ growth} = \% \text{ Parasitemia} - D_0$$

Inhibition percentage was calculated as follows:

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

Where:

D_0 = Percentage parasitemia of infected red blood cell on day 0

Xu = Growth percentage of each sample

Xk = Growth percentage of negative control

Based on the per cent inhibition data, an analysis was made between the test concentration of the per cent inhibition using the SPSS program using probit log analysis to determine the IC_{50} value or the concentration of the test material that could inhibit the growth of parasites by 50%.

Results and discussion

The sample used in this study was Ashitaba leaves which were obtained from Sembalun Village, East Lombok, West Nusa Tenggara. The extraction method was maceration, i.e. to extract active compounds that can dissolve in a solvent-based on the degree of polarity of each solvent (Hans *et al.,* 2010). The distribution of organic solvents that occurs continuously into plant cells results in cell walls and membranes breakdown. This breakdown causes the active compounds in the cytoplasm to dissolve in organic solvents (Sofi & Megawati, 2015). Ashitaba leaf extraction resulted in about 18.6 grams thick brown extract. The qualitative test of the chalcone flavonoid compound was carried out using the Wilstater reagent (Figure 1). The colour change to orange in the sample that had been reacted showed that the Ashitaba leaf sample contained positive chalcone compounds. The colour change in the sample was due to the reduction process of Magnesium (Mg) with concentrated HCl so as to produce complex yellow-orange compounds in chalcones, flavonoids and auronols (Khoplar, 2008).

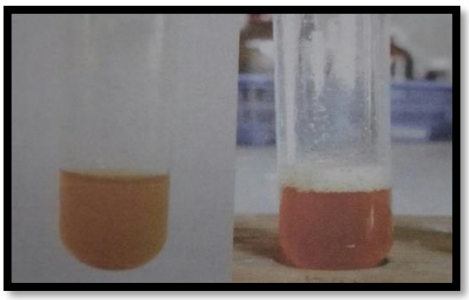

Figure 1: Test of flavonoid compounds with the Wilstater reagent

The test for flavonoid compounds using Bate Smite-Metcalfe reaction changed the colour of the sample to dark red after being reacted and heated, showing that the sample contained flavonoid compounds (Figure 2). This result is in line with research by Amalia (2017), showing that, in the phytochemical screening, Ashitaba plants contain flavonoids (Amalia, 2017).

Figure 2: Test of flavonoids with Bate Smite-Metcalfe reaction

The antimalarial activity test conducted in this study was an *in vitro* test. This *in vitro* test illustrated the antimalarial activity against *Plasmodium falciparum* parasites in the erythrocyte phase because the parasites were grown as if they were in the body's red blood cells. The parasite used in this study was *Plasmodium falciparum* Strain 3D7 which is sensitive to chloroquine (Amalia, 2017). The parasites were incubated for ± 48 hours, followed by the making of a thin blood smear slide. After drying, given 10% Giemsa staining was given. The number of erythrocytes with per cent inhibition was calculated by comparing the number of infected erythrocytes to 1,000 erythrocytes observed under a microscope. The results of the parasite culture observations showed that *Plasmodium falciparum* infecting red blood cells were at the trophozoite stage. Ring-form trophozoite has a ring-like shape with one or two small nuclei and is cytoplasmahalous (Ella, 2017). Antimalarial activity can be determined by calculating the percentage parasitemia obtained in the sample test, resulting in per cent growth, per cent inhibition, mean per cent inhibition, concentration log, and test dose, followed by probit analysis to obtain that the IC_{50} value. The results of the Ashitaba leaf antimalarial activity test can be seen in Table I.

Table I: Data on In Vitro Antimalarial Activity Test Results

Sample	Concentration (µg/ml)	Growth (%)	Inhibition (%)	IC_{50} (µg/ml)
Extract	100	3.23	-	2.09
	10	3.25	-	
	1	0.68	78.95	
	0.1	0.65	80.00	
	0.01	1.05	67.49	
Positive control (Chloroquin)	100	3.23	-	0.007
	10	3.25	-	
	1	0	100	
	0.1	0	100	
	0.01	0.3	90.71	
Negative control (DMSO)		3.24		

Table I shows the percentage parasitemia after the addition of Ashitaba leaf ethanolic extract from the highest concentration to the lowest one. The ethanolic extract of Ashitaba showed the highest per cent inhibition leaves at a concentration of 100 ppm with a per cent inhibition value of 79.47%. In comparison, the lowest one was found at a concentration of 0.01 ppm with a per cent inhibition value of 11.73%. In general, the higher the concentration of the extract given, the higher the per cent inhibition obtained to inhibit the growth of *Plasmodium falciparum*. These results are in line with research by Wardani (2019), showing that the highest

inhibitory effect to the growth of *Plasmodium falciparum* 66.36% resulted from the addition of the highest concentration, i.e. 100 ppm, while the lowest per cent inhibition was found at a concentration of 0.01 ppm with a per cent inhibition of 2.62% (Wardani *et al.*, 2019).

The parasitic resistance values obtained were then analysed using probit analysis. Probit analysis was used to determine the IC_{50} value. The IC_{50} value shows a concentration that can inhibit 50% of cell growth (Achmadi, 2010). The IC_{50} value of Ashitaba leaf extract was 2.09 ppm. The antimalarial activity of Ashitaba leaves fell into the very active category because the IC_{50} value was less than five ppm (Ilhami *et al.*, 2013). A research carried out by Wardani et al. in 2019 stated that there was an antimalarial activity Ashitaba plant, i.e. the stems and roots. Both stems and roots have antimalarial activity as indicated by IC_{50} values of 11.07 ppm and 16.09 ppm, respectively. In addition, the compounds contained in Ashitaba leaves that are believed to inhibit the growth of parasites are chalcone compounds, which belong to the flavonoid class (Wardani *et al.*, 2020).

Conclusion

Based on the results of the research that has been done, it can be concluded that the ethanolic extract of Ashitaba leaves has antimalarial activity because it has an IC_{50} of 2.09 ppm, which falls into the very active category in inhibiting 50% of the growth of the parasite *Plasmodium falciparum* strain 3D7.

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Conflict of Interest

The authors declare no conflict of interest.

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