In Vitro Effect of 96% Ethanol Extract of Bitter Herbs (Andrographis paniculata Nees) on Heme Detoxification Process of Plasmodium falciparum Parasites

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Abstract: Malaria still becomes a global health problem especially in tropical countries and developing countries. The number of malaria infections in Indonesia is still high and it becomes endemic disease in some areas. The high rate incidence of malaria can be caused by the emergence of malaria parasite strains that are resistant to anti malarial drugs. Various efforts have been made to reduce the incidence of malaria, one of which was to develop a new medicinal compounds from natural ingredients such as 96% ethanol extract of bitter herbs (Andrographis paniculata Nees). This herb has been proven to have antimalarial activity both in-vitro and in-vivo from previous studies. This study aims to determine the effect of 96% ethanol extract of bitter herbs on heme detoxification process of Plasmodium falciparum through the inhibition of β-hematin formation (synthetic heme). This research is a laboratory experimental research that was conducted in Malaria Laboratory of Institute of Tropical Disease (ITD) University of Airlangga Surabaya. The results showed that 96% ethanol extract of bitter herbs inhibits the formation of β-hematin equal to 61.07 ± 4.69%. It can be concluded that 96% ethanol extract of bitter herbs (Andrographis paniculata Nees) are able to inhibit heme detoxification process of Plasmodium falciparum parasites.

1 INTRODUCTION

96% ethanol extract is a natural herb obtained from bitter plants (Andrographis paniculata Nees / Sambiloto). This traditional medicinal plant that has long been used empirically as an anti-malarial drug (Widyawaruyanti, 2009). The compound substances are contained with Sambiloto herbal plants include diterpenoid lactone, flavonoid, xanton and Androgapholide which are the main active substances of this plant. The level of Androgapholide in the form of ethanol extract 96% is ± 14.91% (Okhuarobo et all, 2014; Safitri EY, 2007).

96% ethanol extract of Sambiloto herb are obtained from extraction process of Sambiloto powder through maceration process with its solution is 96% ethanol to enable it to draw a range of compounds contained in Sambiloto plant (Widyawaruyanti, 2009). Earlier research has indicated that 96% ethanol extracts of Sambiloto herbs are active as antimalarial drug against Plasmodium falciparum in vitro, and are also active Against Plasmodium berghei in-vivo (Widyawaruyanti et al, 2014; Rahman et al, 1999). Other studies show that 96% ethanol extracts of Sambiloto herbs were found to have antimalarial activity at medium-to-moderate categories with inhibitor concentration value (IC) 2,287 µg/ml that inhibits the development of schizont stage (Schizontocide) (Resi, 2013; Widyawaruyanti et al, 2015).

One of the important work targets of antimalarial drugs in killing malaria parasites is by inhibiting the process of heme detoxification from developing into hemozoin crystals. This is a vital process for the parasite and has been validated as one of the antimalarial work targets. Therefore it is necessary to study the effect of 96% ethanol extract of Sambiloto herbs in inhibiting the process of heme detoxification of Plasmodium parasite to figure out
the mechanism of 96% ethanol extract of Sambiloto herbs in killing parasites.

2 RESEARCH METHODOLOGY

This is a laboratory experimental research. The antimalarial herb 96% ethanol extract Sambiloto herbs at dose 100 μg / ml is incubated with hemin chloride and then its effect is observed on heme detoxification process by using spectrophotometer. The sample used in this study was hemin chloride which is a synthetic pigment obtained from Sigma®.

The inhibition of the P. falciparum heme parasite detoxification process is carried out by synthetically testing the inhibition of β-hematin formation. In this test, the active antimalarial compound of 96% ethanol extract of Sambiloto herbs with the 100 μg / ml concentration obtained from the ITD Science and Technology Research Partnership for Sustainable Development (SATREPS) Laboratory was incubated in acetate buffer with α-chlorohemin (Sigma) solution for 24 hours, with trial of duplication.

2.1 Preparing the Test Substance of 96% Ethanol Extract of Sambiloto Herbs

The 96% ethanol extract of Sambiloto herbs as the test substance in this study was firstly prepared through the following steps:

- The extract is to be weighed 10 mg of test substance, dissolved in 100 μl DMSO to obtain solution concentration of 100.000 μg / ml as stock solution
- Measure as much as 10 μl of stock solution with pipette then add a complete medium until the volume reaches 500 μl which results in the solution concentration of 2000 μg / ml (Solution A)
- Measure as much as 10 μl of solution A with pipette then add a complete medium until the volume reaches 500 μl which results in the solution concentration of 100 μg / ml (Solution B)

2.2 Procedure of synthetic heme detoxification inhibition Test (β-Hematin Inhibition Assay) (Tekwani BL and Walker LA, 2005)

The inhibition test of heme detoxification process is undertaken through the following steps:

- Turn solid natrium acetate into 8M natrium in warm water. Do the mixing continuously at 370°C in waterbath
- Add 8 M acetic acid until it reaches pH 5.0 value. The addition of acetic acid is to be done at 370°C, then incorporate a total of 100 μl of the solution in the falcon tube.
- Add the 96% ethanol extract test substance of 50 μl with 100 μg / ml concentration in DMSO in a falcon tube containing the acetate buffer solution.
- Add a total of 50 μl of hemin chloride 8mM (Sigma) in DMSO to the falcon containing the test substance and acetate buffer solution.
- Undertake incubation for 24 hours at 35°C with two replications.
- Centrifugalize β-hematin and insoluble hematin (in the form of pellet) at 3000 rpm for 20 minutes at a temperature of 22 °C, and discard the supernatant.
- Add 200 μl DMSO to the obtained pellet to dissolve unreacted hematin, then re-centrifugalize it and discard the supernatant.
- Add 200 μl NaOH 0.2M to the pellet to convert β-hematin into a soluble hematin alkaline, then make each sample of alkaline hematin in dilution series with Aqua bidest.
- Measure absorbance using a spectrophotometer at a wavelength of 414 nm.
- Calculate the effect of the test substance on the production of β-hematin and compare it with the negative control. The negative control used in this study was 50 μl of DMSO solution.

In this study, the data analysis was undertaken descriptively by comparing the group that has been given 96% ethanol extract of sambiloto herbs with the negative control group.

3 RESULTS AND DISCUSSION

The Plasmodium parasite will consume hemoglobin from erythrocyte cells as one of its nutrients for its survival. The stage at which hemoglobin is mostly consumed is the tropozoit stage. Being in erythrocytes, the parasite endocytoses a number of nutrients that are mostly hemoglobin coming from erythrocyte cytosol.

73
Endocytosis occurs through the process of invagination of parasitic plasma membranes known as sitostoma. Once the endocytosis process is complete, the neck of sitostoma is removed to release the double membrane of the endocytic vesicle that contains the cytoplasm of red blood cells.

The digestion process of erythrocyte cytoplasm is initiated by proteolytic gastrointestinal enzymes in double membranes vesicular transport. The double membrane vesicular transport is thought to originate from the invagination of the parasitic plasma membrane as the outer layer and the parasitophorous vacuoles membrane as the inner layer.

The result of host cytoplasm endocytosis (80% hemoglobin) will be transported by vesicle transport into the food vacuole and degraded proteolitically into heme and globin in the food vacuole namely an oxygen-rich lysosome-like organelle at the pH between 5-5.5 (Hong-Chang Z et al, 2009; Hoppe HC, 2004).

The Plasmodium parasite consumes only globin as its nutritional source, while the heme is released. Free heme is toxic to the parasite and has the ability to lyse the parasitic membrane of Plasmodium. Therefore the parasite must detoxify the heme through certain mechanisms; one of which is by converting free heme into insoluble crystal hemozoin (Egan TJ, 2007).

The effects of Plasmodium parasite’s heme inhibition can be known through β-hematin formation inhibition test (β-Hematin Inhibition Assay). β-hematin has a structure and spectrum identical to hemozoin, in which case the β-hematin formation inhibition test may be used to determine the effect of antimalarial drugs on the detoxification process of heme parasite P. falciparum.

β-hematin can be formed in vitro in acidic atmosphere (acetate buffer solution pH 5.0) of hematin or hemin chloride. Hemin chloride will react to acetate buffer and form an insoluble β-hematin after 24-hour incubation at 35°C. The administration of NaOH solution in the β-hematin pellet will convert it into alkaline hematine whose absorbance can be measured using UV.8 spectrophotometer (Tekwani BL and Walker LA, 2005).

The result of calculation on percentage of β-hematin formation inhibition in table 1 shows that in the group which was given 96% ethanol extract of Sambiloto herbs, β-hematin formation activity was inhibited with the average percentage of β-hematin formation inhibition of 61.07 ± 4.69%.

Frolich et al (2005) suggests that compounds with β-hematin formation inhibition greater than 60% are stated to have good potential as β-hematin inhibitors.

Compounds with β-hematin formation inhibitions smaller than 40% are claimed to be weak inhibitors on the formation of β-hematin.

Therefore, it can be stated that 96% ethanol extract of the Sambiloto herbs has good potential as an β-hematin formation inhibitor.

This result is supported by a research conducted by Widyawaruyanti et al (2015) which states that 96% ethanol extract of Sambiloto herbs is known to have activity of inhibiting the process of detoxification of heme into crystalline polymer hemozoin through observation of food vacuoles parasit P.falciparum with TEM (Transmission Electron Microscope) microscope. It was mentioned that, in the parasite which was given 96% ethanol extract Sambiloto herbs, it appears that the crystal hemozoin was undergoing fragmentation.

On the contrary, in the control group, it appears that the woven crystals are still clear and transparent (Widyawaruyanti, 2015).

The aforementioned description shows that ethanol extract of Sambiloto herbs has an inhibitory effect on the detoxification process of heme parasite Plasmodium falciparum and is one of ethanol extract work target of 96% Sambiloto herbs. This inhibition process can minimize the pathogenesis of malaria and also inhibit the formation of hemozoin in the liver and spleen of malaria patients.

<table>
<thead>
<tr>
<th>No</th>
<th>Substance</th>
<th>R</th>
<th>Absorbance (414 nm)</th>
<th>% β-hematin formation</th>
<th>% β-hematin inhibition</th>
<th>% mean inhibition of β-hematin</th>
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<tr>
<td>1</td>
<td>Control</td>
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</table>
4 CONCLUSIONS

In conclusion, 96% ethanol extract of Sambiloto herbs have the activity of inhibiting the process of heme parasite Plasmodium detoxification.

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REFERENCES


