In Vitro Study: Antimalarial Activity of Rivet Sea Cucumber Extract (Holothuria atra) With Ethyl Acetate Solvent Against Plasmodium falciparum

Zuhro Putri Febrianti¹, Prawesty Diah Utami², Verna Biutifasari³

¹Faculty of Medicine Hang Tuah University, Surabaya, Indonesia
²Parasitology Departement, Faculty of Medicine Hang Tuah University, Surabaya, Indonesia
³Pathology Clinic Departement, Faculty of Medicine Hang Tuah University, Surabaya, Indonesia

Email¹: prawesty.diah@hangtuah.ac.id

Abstract

Indonesia is experiencing a decreased cure rate of malaria caused by the species Plasmodium falciparum. Alternative medicine, in this case, uses marine biota, namely lollyfish (Holothuria atra), which has a lot of active compound content; The use of marine biota is stimulated by reports of resistance to artemisinin-based combination therapy (ACT). The purpose of this study was to find out the antimalarial activity of Holothuria atra extract with ethyl acetate solvent at doses of 0.01; 0.1; 1; 10; and 100 µg/ml against Plasmodium falciparum in vitro. Extract lollyfish using the maceration method. The lollyfish powder will be dissolved with ethyl acetate solvent at doses of 0.01; 0.1; 1; 10; and 100 µg/ml against Plasmodium falciparum. Antimalarial activity testing is measured through 4 parameters, namely the measurement of parasitemia rates, % parasite growth, % inhibitory rate, and IC50. Antimalarial activity with ethyl acetate solvent belongs to a very active category with an IC50 value of 1.52 µg/mL. These results can kill the P. falciparum parasite with a percentage that reaches 0%. A decrease in the percentage of growth occurs when the dose given is too low so that the parasite can grow and survive. The result of higher concentrations of extracts will have a higher percentage of resistance to the growth of parasites. The potential for antimalarial activity in lollyfish extract is influenced by the presence of active content such as alkaloids, flavonoids, catechins, and pyrogallol owned by marine biota animals. This study suggests that lollyfish (H. atra) can be developed as an alternative treatment for malaria.

Keywords: Holothuria atra, antimalarial, Plasmodium falciparum, in vitro

INTRODUCTION

Malaria is an infectious disease of the Plasmodium parasite, transmitted through the bite of the Anopheles sp that has various symptoms such as cold chills and persistent fever because the area is too dense with poor sanitation that can facilitate the transmission of the disease. People who migrate to endemic areas can facilitate the spread of malaria (1–3). The three states attributed for 99.5 percent of all malaria infections in Southeast Asia, with India providing the most incidents (87.9%), Indonesia (10.4%), and Myanmar (1.2%). There are five species of malaria in humans (P. falciparum, P. vivax P. ovale, P. malariae, and P. knowlesi) (4), but Plasmodium falciparum infection can result in severe circumstances and fatality (5–7).

ACT resistance has been identified in numerous countries, including Cambodia, Myanmar, Thailand, Vietnam, and the Democratic Republic of Laos. The role of parasite molecular markers in the occurrence of resistance due to the occurrence of mutations in parasite receptors associated with delayed clearance of parasites. Failure of ACT therapy to ensure treatment is productive or not, WHO has advised conducting regular monitoring at least once every 24 months in a row to detect changes that occur and efficacy in therapy (8). Due to artemisinin resistance in P. falciparum malaria has spread globally around the world, therefore it takes alternative antimalarial treatments, one of which comes from marine biota (9,10).

Indonesia's vast territorial waters make it a source of marine life that has a
therapeutic effect on various diseases. So coastal areas with the potential for marine life can be used as an alternative medicine for a new regimen for malaria (11-13). Sea cucumbers (*Holothuria atra*) are healthy food ingredients that contain various physiologically active substances, including vitamins A, C, B1, B2, B3, iron, magnesium, calcium, zinc, chondroitin sulfate, and saponin glycosides. Sea cucumber extract also produces effects to inhibit tumor cell growth and as an antiprotozoal activity (14,15). *Holothuria atra*, also known as sea cucumber or lollyfish, is a species of sea cucumber commonly found in eastern Indonesia's waters. This species is most commonly found in coral reefs and seagrass meadows (16,17). Sea cucumber extract (*Holothuria atra*) with ethyl acetate solvent in phytochemical tests showed positive to contain a group of alkaloid compounds, steroids, and saponins. This research is important to explore a new antimalarial regimen.

**METHODS**

This research was in vitro study with a posttest-only control group design. The experimental group was divided into three groups, of which (1) The negative control group included ten culture media of *P. falciparum* without antimalarial drugs or *H. atra* extract; (2) The positive control group included ten culture media of *P. falciparum* with antimalarial drug administration (chloroquine) but no extract administration; and (3) A treatment group included ten culture media of *P. falciparum* with ethyl acetate extract *H. atra* administration, but no antimalarial administration (18).

The study was conducted in 4 stages: preparation, extraction, in vitro antimalarial activity examination (by measuring levels of parasitemia, inhibitory rate, and inhibitory concentration 50/IC50), and descriptive – statistical analysis.

**Preparation:**

The research sample was *P. falciparum* (3D7 type, chloroquine-sensitive strain) culture media obtained from the ITD/ Institute Tropical Disease, Medicine Faculty of Airlangga University.

Sea cucumber, *H. atra* was obtained from the waters of Madura Sabuntan Village Saeken Subdistrict and extracted at Veterinary Faculty Airlangga University. *H. atra* taxonomic test was conducted in the Lab. MIPA ITS. The ethics committee of Hang Tuah University's medical faculty has approved that this research be carried out.

**Extraction Process**

The sample of sea cucumber (*H. atra*) is extracted by the maceration technique by drying the previously small cut sample first. Samples that have been dried are smoothed to widen their surface so that samples and solvents can give maximum results. *H. atra* powder will be dissolved with ethyl acetate solvent for 24 hours. The results are collected and evaporated with a vacuum rotary evaporator and put into an airtight container and stored in the refrigerator until use (17,19,20).

**Examination of Antimalarial Activity**

The antimalarial properties were evaluated using the Trager and Jansen method with Giemsa staining. The extract concentration dose used in this study was 0.01; 0.1; 1; 10; and 100 μg/ml. The test material was put into 24 disposable microwell plates with 1% parasite breed suspension and 5% hematocrit. The culture is inserted into the candle jar and incubated for 48 hours at a temperature of 37°C. Culture results are made preparations of thin blood removal with 20% Giemsa staining. Removal of dried blood is carried out by the microscopic examination with a magnification of 100x. Observation of the percentage of parasitemia and percentage of inhibition is done after the microscopic examination (19–21).

1. The first parameter used to assess antimalarial activity is parasitemia level. The calculation formula for the level of parasitemia levels is:
2. The second parameter used to assess in vitro antimalarial activity is an inhibitory percentage. The calculation formula of inhibition percentage is:

\[
\frac{\% \text{ Inhibition}}{100} = \frac{X_u - X_k}{X_k} \times 100\%
\]

Xu represents the percentage of parasitemia tests and Xk is the percentage growth on negative controls.

3. The last parameter to measure antimalarial activity in vitro is to calculate IC50 using probit statistical analysis.

Table 1-1 Percentage of parasitemia levels before and after administration of H. atra extract with ethyl acetate solvent against P. falciparum

<table>
<thead>
<tr>
<th>Concentration (µg/mL)</th>
<th>Group Research</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>G1</td>
</tr>
<tr>
<td>100</td>
<td>6.73</td>
</tr>
<tr>
<td>10</td>
<td>6.73</td>
</tr>
<tr>
<td>1</td>
<td>6.73</td>
</tr>
<tr>
<td>0.1</td>
<td>6.73</td>
</tr>
<tr>
<td>0.01</td>
<td>6.73</td>
</tr>
</tbody>
</table>

Group 1 was a negative control group that got the same results because negative control was not treated without the administration of antimalarial drugs or H. Atra extract. Group 2, namely positive control with the administration of antimalarial drugs (chloroquine), showed low levels of parasitemia at a dose of 10 µg / ml, which is 2.92%. Group 3 showed the lowest levels of parasitemia at a dose of 10 µg/ml at 2.82% so the levels of group 3 parasitemia were lower than group 2 at 10 µg/ml.
Group 1 on negative control obtained a fixed result because no treatment was done. Group 2 on positive control did not measure the growth of parasites at doses of 100 μg/mL because the administration of chloroquine dose of 10 μg/mL has been able to suppress parasites close to zero (0). Group 3 on the growth of P. falciparum with an extract dose of 100 μg/mL showed results (0) in the absence of parasite growth at that dose so a large concentration dose provides a low percentage of parasite growth even if parasites cannot grow at that dose.

Table 1-2 Percentage growth of P. falciparum parasite

<table>
<thead>
<tr>
<th>Concentration (µg/mL)</th>
<th>Group Research</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>G1</td>
</tr>
<tr>
<td>100</td>
<td>5.73</td>
</tr>
<tr>
<td>10</td>
<td>5.73</td>
</tr>
<tr>
<td>1</td>
<td>5.73</td>
</tr>
<tr>
<td>0.1</td>
<td>5.73</td>
</tr>
<tr>
<td>0.01</td>
<td>5.73</td>
</tr>
</tbody>
</table>

Group 1 on negative control obtained a fixed result because no treatment is done. Group 2 on positive control showed that at doses of 10 μg/mL, the percentage of inhibition reached 92.46%, which was close to 100% so that at doses of 100 μg/mL, no more measurements were taken. The percentage inhibition of group 3 is greatest in extract H.atra ethyl acetate solvent with a concentration of 100 μg/mL, which is 100 percent. According to all the data, the higher the concentration of H. atra extract, the greater the inhibitory activity.

Table 1-3 Inhibitory rate P. falciparum

<table>
<thead>
<tr>
<th>Concentration (µg/mL)</th>
<th>Group Research</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>G1</td>
</tr>
<tr>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>0.1</td>
<td>0</td>
</tr>
<tr>
<td>0.01</td>
<td>0</td>
</tr>
</tbody>
</table>
Chloroquine inhibition ability on positive control indicates that a dose of 10 μg/mL can inhibit the development of parasites with achievements close to 100%. This result suggests that chloroquine inhibition ability was greater when administered at doses of 10 μg/mL compared to the group that got extract. The increase in the dose of the concentration of extracts tested, the greater the inhibitory activity. It is difficult to compare the results of this study with other similar studies, as no studies have been found exploring the antimalarial effects of H.aatra (17,20).

Observations of the growth of parasites on negative controls showed the highest percentage. This is thought to occur due to the natural response of the P. falciparum parasite to defend itself in environmental conditions that are not suitable for its survival, so the parasite seeks to speed up the process of its growth (23,24). The negative control group contained only media containing erythrocytes and P.falciparum strains of 3D7 without the administration of drugs or extract ingredients that could inhibit its growth. Because of this, the growth in the control group showed the highest value among the other two groups (17,20).

This research used the parasite's growth rate to calculate the percentage growth of P. falciparum after using H. atra extract with ethyl acetate solvent at various doses. The larger the dose of concentration tested, the smaller the growth of the parasite P. falciparum. A decrease in the growth percentage occurs when the dose given is high so that the parasite can’t survive and grow. The results of the resistance percentage measurement showed that the highest inhibition effect on sea cucumber extract with ethyl acetate solvent was obtained at a concentrated dose of 100 μg/mL with inhibition reaching 100%.

Chloroquine inhibition ability on positive control indicates that a dose of 10 μg/mL can inhibit the development of parasites with achievements close to 100%. This result suggests that chloroquine inhibition ability was greater when administered at doses of 10 μg/mL compared to the group that got extract. The increase in the dose of the concentration of extracts tested, the greater the inhibitory activity. It is difficult to compare the results of this study with other similar studies, as no studies have been found exploring the antimalarial effects of H.aatra (17,20).

Probit analysis results on IC50 values used to inhibit the growth of P. falciparum with ethyl acetate solvent of 1.52 μg/mL. This means that H.aatra extract has strong antimalarial potential (IC50 < 5 g/mL). Chloroquine has an IC50 value of 0.04 μg/ml meaning that chloroquine produces a very active antimalarial potential compared to ethyl acetate solvent H.aatra extract.

DISCUSSION

Calculation of parasitemia levels was done to determine the difference in the number of P. falciparum parasites at each concentration tested. The results of processing data in table 1-1 showed that the higher the dose of H.aatra extract was inversely proportional to the level of parasitemia because the larger the dose of the extract had the greater ability to suppress parasite growth (17,22). At a dose of 100 μg/mL extract, it showed the highest suppression of parasitemia levels (0.29 %), the same as the G2 group that received chloroquine (with a dose of 100 μg/mL, it was able to suppress the parasite level until it was undetectable or 0%) (17,23).

This research used the parasite's growth rate to calculate the percentage growth of P. falciparum after using H. atra extract with ethyl acetate solvent at various doses. The larger the dose of concentration tested, the smaller the growth of the parasite P. falciparum. A decrease in the growth percentage occurs when the dose given is high so that the parasite can’t survive and grow. The results of the resistance percentage measurement showed that the highest inhibition effect on sea cucumber extract with ethyl acetate solvent was obtained at a concentrated dose of 100 μg/mL with inhibition reaching 100%.

Chloroquine inhibition ability on positive control indicates that a dose of 10 μg/mL can inhibit the development of parasites with achievements close to 100%. This result suggests that chloroquine inhibition ability was greater when administered at doses of 10 μg/mL compared to the group that got extract. The increase in the dose of the concentration of extracts tested, the greater the inhibitory activity. It is difficult to compare the results of this study with other similar studies, as no studies have been found exploring the antimalarial effects of H.aatra (17,20).

Observations of the growth of parasites on negative controls showed the highest percentage. This is thought to occur due to the natural response of the P. falciparum parasite to defend itself in environmental conditions that are not suitable for its survival, so the parasite seeks to speed up the process of its growth (23,24). The negative control group contained only media containing erythrocytes and P.falciparum strains of 3D7 without the administration of drugs or extract ingredients that could inhibit its growth. Because of this, the growth in the control group showed the highest value among the other two groups (17,20).

Positive controls in this study used chloroquine. Chloroquine is a group of 4-aminoquinoline compounds used as antimalarial drugs. Chloroquine can inhibit the growth of parasites through its schizonticide mechanism and blood gametocytocidal in all types of human plasmodium (20,25). The mechanism of action of chloroquine is the acidic nature of chloroquine in the food vacuole that can increase the pH of organelles so that the metabolism of parasites is disrupted and toxic substances contained in

Table 1-4 Inhibitory Concentration extract of H. atra against P. falciparum parasites

<table>
<thead>
<tr>
<th>Active Compounds</th>
<th>Extract Dose (µg/mL)</th>
<th>IC50 (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H atra with ethyl acetate solvent</td>
<td>8.16</td>
<td>25.70</td>
</tr>
<tr>
<td>Klorokuin</td>
<td>53.68</td>
<td>69.12</td>
</tr>
</tbody>
</table>
chloroquine will poison the vacuoles thereby inhibiting food intake and parasites starve to death (26,27).

Interventions conducted in this study used an extract of sea cucumber *H. atra* with ethyl acetate solvent. Biodiversity in marine waters is much higher than on land. Many potential marine species are used as medicine and are very beneficial for humans. Sea cucumber or also called *Echinoderms* is a marine animal with invertebrate phylum that has many pharmacological benefits (28,29). Compounds from sea cucumbers have pharmacological activities including antimalarial, antitumor, anticoagulant, antimicrobial, antioxidant, antiviral, and antifungal (30–32).

The body wall of sea cucumber contains polysaccharides consisting of fucan sulfate and *Fucosylated Chondroitin Sulfates* (FuCS), which have pharmacological activity as anticoagulants and antimicrobial activities (31,33). Phytochemical test of antifungal activity of *H. atra* extract with positive ethyl acetate solvent contains triterpene glycoside compounds that can be used as antifungals (34–36). Phenolic compounds and alkaloids in the internal organs of *H. atra* can also act as antioxidants. Saponin compounds act as antibacterials with their mechanism of work that kills bacteria by removing proteins and enzymes from inside the cell (37–39). Sulfate saponin compounds in sea cucumbers can also act as antitumors by inhibiting the proliferation of colorectal cancer cells (40,41).

Identification of active compounds in *H. atra*, which are flavonoid, phenolic, and alkaloid compounds has been done in phytochemical tests and is suspected to have antimalarial activity (23,42,43). Alkaloids are active nitrogenous compounds derived from animals and plants. Alkaloids have been successfully used for the treatment of malaria. Alkaloids can inhibit the formation of proteins, DNA, and RNA *P. falciparum* by inhibiting the enzyme *Plasmodium falciparum Dihydrofolate Reductase* (PDHFDH) which synthesizes RNA and DNA in parasites (44–46). Hetero flavone compounds of the flavonoid group have the highest and most active antimalarial activity due to the lowest IC50 values. One group of flavonoids, chalcone, can lower hemolysis levels from erythrocytes infected with *P. falciparum*. Catechins and pyrogallol are two phenolic compounds that have antimalarial properties. In the trophozoite and schizont phases, catechins can prevent *P. falciparum* from growing, so that merozoites cannot come out to infect erythrocytes (43,47). Pyrogallol resulting from the hydrolysis of tannins from gallic acid can inhibit enzymes in *P. falciparum* parasites, the enzyme *Dihydrofolate Reductase* (DHFR) that synthesizes folate so that the growth of parasites will be disrupted (48,49). This is with various active compounds contained in *H. atra* has the potential as an antimalarial that can suppress the development of *P. falciparum* in line with the results of this in vitro study.

The chance of *H. atra* extracts with ethyl acetate solvent as an alternative malarial drug can be proven by having very active antimalarial activity with its IC50 value. Ethyl acetate solvent *H. atra* with a concentrated dose of 100 µg/mL can kill the parasite *P. falciparum* with a growth percentage of 0%. The semipolar solvent ethyl acetate has been shown to have antimalarial activity (17,33,50) and further research is needed to find out and explore compounds in *H. atra* extract that have antimalarial activity.

**CONCLUSION**

In vitro studies show that the extract of sea cucumber (*Holothuria atra*) with ethyl acetate solvent at doses of 0.01; 0.1; 1; 10 and 100 µg/mL has antimalarial against *P. falciparum*. This is evidenced by a decrease in the percentage growth of parasites in the administration of ethyl acetate extract *H. atra* compared to negative controls. The greater the dose of extract concentration, the higher the percentage resistance to the growth of parasites *H. atra* extracts with ethyl acetate solvent was shown to produce highly active antimalarial activity with an IC50 value of 1.52 µg/mL. Although the value is still lower than...
IC50 chloroquine, this is likely due to the use of chloroquine-sensitive *P. falciparum* strains.

ACKNOWLEDGEMENT

The authors of the article express their gratitude to the Faculty of Medicine at Universitas Hang Tuah and the Laboratory Institute of Tropical Disease at Airlangga University for their contributions to the in vitro research.

REFERENCES


13. Hidayati AR, Widyawaruyanti A,


