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# ORIGINAL ARTICLE

## In-vivo antimalarial activity of *Holothuria scabra* simplicia in *Plasmodium berghei-infected* mice

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## ABSTRACT

**BACKGROUND**

Malaria remains one of the major causes of death worldwide. *Holothuria scabra* has a high nutritional content and many biological effects. The development of alternative anti-malarial drugs is necessary, considering that resistance to the newest anti-malarial drugs has been reported. This research study aimed to determine the anti-malarial effects of *Holothuria scabra* in mice infected with *Plasmodium berghei.*

**METHODS**

This was a post-test only control group study conducted on 24 Balb/c mice. Peter's four-day suppressive test was employed to ascertain the claimed anti-plasmodial effect of the simplicia. Following inoculation with *P. berghei*, all 24 mice were infected and randomized into 4 groups, namely 3 treatment groups and 1 control group. The control group was given carboxymethyl cellulose, two of the treatment groups were given doses of 10 and 100 mg/kg BW *Holothuria scabra*, respectively, using Peter's four-day suppressive test, while the remaining treatment group received a dose of 100 mg/kgBW using the prophylactic method. Data were analyzed using One way ANOVA.

**RESULTS**

The results showed by using both the four-day suppressive test and the prophylactic method, that *Holothuria scabra* has antimalarial activity. *Holothuria scabra* at a dose of 100 mg/kg BW was significantly effective in decreasing the percentage of parasitemia (p=0.000) and tended to inhibit the growth of *Plasmodium berghei* in mice (p=0.054).

**CONCLUSION**

This study demonstrated that *Holothuria scabra* possesses anti-plasmodial activity in mice. Hence, the sea cucumber could serve as a potential source of a newer antimalarial agent.

**Keywords:** Malaria, *Holothuria scabra*, anti-malarial, *Plasmodium berghei*, parasitemia, mice

## INTRODUCTION

Effective antimalarial drugs are the key to malaria control and elimination, therefore the effectiveness of current standard antimalarial drugs must be continuously monitored. The standard drug treatment for malaria currently used is artemisinin-based combination therapy (ACT).[1] However, resistance to ACT has been reported in the Greater Mekong subregion, namely the Mekong River Basin in Southeast Asia.[1] Areas that are the locus or focus of resistance include the border areas of Thailand-Cambodia, Thailand-Myanmar, Vietnam-Laos, and Vietnam-Cambodia. In fact, recently it has been reported that ACT resistance has become the center of attention in sub-Saharan Africa, especially Angola.[2] The latest WHO report states that Rwanda and Uganda have also experienced resistance to both artemisinin and the combination therapy. [3]

*Holothuria* spp. are spiny-skinned invertebrates in the phylum Echinodermata, which is a commercial commodity that is quite important for health and is consumed by the public. There are 200 species of holothurians of which 75 species originate from shallow waters.[4]

Of all the *Holothuria* species, *Holothuria scabra* is the most intensively studied species. Sea cucumbers or sandfish belong to the phylum Echinodermata, order Aspidochirota, family Holothuroidea and have been discussed in the literature since the 19th century. The reason *H. scabra* is an important species is because it is widely distributed in abundant numbers throughout the Indo-Pacific region. *H. scabra* also has a high commercial value on the market; currently it is widely sold in the form of dried products, with *H. scabra* being the only holothuroid species that has been mass-produced at breeding sites.[4]

*Holothuria scabra* contains phenols, quercetin, saponins, tannins, flavonoids, and antioxidants such as vitamin C.[5,6] Marine-derived components containing (E)-oroidin and (E)-oroidin trifluoroacetic acid (TFA) salt are known to have antimalarial activity through the FabI inhibition mechanism.[7] The research studies of Febrianti et al.[8] and Ramadania et al.[9] have found that *H. atra* has antimalarial effects in vitro, probably due its components such as alkaloids, flavonoids, catechins, and pyrogallol.[8] Other sea cucumbers such as *Ludwigothurea grisea*, *Holothuria scabra*, and *Isostichopus badionotus* have been extensively studied for the antiplasmodial capacity of heparin-like sulfated polysaccharides.[10] Fucosylated chondroitin sulfate (FCS) isolated from *H. grisea* showed pronounced antimalarial effects via inhibition of plasmodium development.[10]

*Holothuria spp* has numerous species and characteristics. *Holothuria grisea* is cylindrical in shape with a flat belly, can reach 30 cm in length, has a basic coloring ranging from red, orange, to yellow, with brown spots. The animals are generally found in shallow tropical waters in the Atlantic Ocean from Florida to Southern Brazil and West Africa. Meanwhile, *Holothuria atra*, often called the blood sea cucumber, has a round body cross-section, a ventral side that tends to be flat, and a round anal opening. The body color is black, and the skin is soft and thick. It is found in areas with a substrate of coarse sand and its body is covered in fine sand. *Holothurian scabra* is known as the sand sea cucumber, its body shape is elongated and cylindrical, and it is relatively fat. The color of the dorsal side varies from brownish gray to black, with dark wrinkles throughout the body and small black papillae from tip to tip.[8,10] Nevertheless, the effectiveness of *H. scabra* for malaria treatment have not been scientifically proven. Therefore, this study assessed the effectiveness of *H.scabra* simplicial in the reduction and prevention of blood plasmodial levels in mice infected with *P. berghei*.

## METHODS

### Research design

This was an experimental laboratory study using an animal model with a post-test only control group design. This study was conducted at the Integrated Biomedical Laboratory Unit, Faculty of Medicine, Universitas Udayana, Denpasar, Bali from September until November 2023.

### Preparation of *H. scabra*

The sea cucumbers used in this research were dried sea cucumbers purchased from Gresik, East Java. Chemicals used in this study were phosphate-buffered saline (PBS), distilled water, carboxy-methyl cellulose (CMC), Giemsa stain, and absolute methanol (MERK). As a preliminary, the dried sea cucumbers were initially boiled such that the texture of the meat became supple again. The procedure for making *H. scabra* preparations was based on that of Herliany et al.[11] The boiled sea cucumbers were weighed according to the calculation of the dose required for all mice in the

treatment group during the study period. The volume of *H. scabra* simplicia should not exceed the capacity of the mouse stomach, therefore 1% Na-CMC (sodium carboxy methyl cellulose) suspension was used, while the control group was given 1% Na-CMC (sodium carboxy-methyl cellulose) suspension without *H. scabra*. The 1% Na-CMC suspension was made by dissolving 1.0 g of Na-CMC in 100 ml of distilled water and then stirring with a magnetic stirrer until homogeneous. The *H.scabra* suspension was made by dissolving sea cucumbers that had been dissolved in boiling water and weighing them according to the desired concentration, namely 1%.

**Experimental animals**

The research animals were 8-week-old male Balb/ c mice weighing 25-30 grams, obtained from the Bioscience Laboratory, Universitas Udayana (Bali, Indonesia). All mice were acclimatized for one week at controlled temperature (20±5°C) and humidity (55±15%) in a cycle of 12 hours light and 12 hours darkness. The sample size was determined by the Federer formula: (t-1) (r-1) >15 (t=number of treatments; r=number of replications); (4-1)(r- 1) >15; 3(r-1)>15; r>5. In anticipation of a drop- out rate of 10%, a total sample size of 24 was obtained for the four groups.

**In vivo antimalarial tests**

In vivo antimalarial tests were carried out using the *P. berghei* obtained from the Faculty of Medicine, Brawijaya University. Parasite stocks were maintained by continuous reinfection of mice. The in vivo procedures in this study were carried out according to Peter's 4-day suppressive test method and repository test method adopted from the Peter's prophylaxis method.[12] All 24 mice were infected with *P. berghei*, then 3 hours after the parasite infection, the mice were randomly assigned to the 3 treatment groups consisting of 6 mice per group. The control group was given 1% CMC, the treatment groups P1 and P2 were given doses of *H. scabra* at 10 and 100mg/kg BW, while group P3 was given 100 mg/kgBW of *H. scabra* using the prophylaxis method. Evaluation of the prophylactic activity of *H. scabra* that was adopted in this study consisted of giving the simplicia from day 0 to day 7, then a blood smear was made from each mouse 72 hours after treatment, and the parasitemia and percentage of growth inhibition were calculated according to Adetutu et al.[13]

Parasitemia growth and inhibition in this study were calculated using formulas for parasitemia percentage and percentage of parasite growth inhibition. To determination the percentage of blood cells infected with malarial parasites, the slides were carefully observed under the light microscope using the x100 objective with immersion oil in 5 different fields of view on each slide. The assessment of in vivo antimalarial activity was performed using a 4-day suppressive test to evaluate the schizonticidal activity of the simplicia.[12] The parasitemia suppression (PPS) or parasitemia inhibition percentages were calculated using the formula described below: percent parasitemia (PP) in the negative control group is subtracted from the mean PP in the treatment group and the result is divided by PP in the negative control group.

**Statistical analysis**

For statistical analysis, first a normality test of data distribution was conducted using SPSS software (v22.0). The analysis of variance (ANOVA) followed by multiple comparison test using Tukey Honest Significant Difference (HSD) was conducted if the data was normally distributed, while the Kruskal-Wallis test was used for analysis if the data distribution was not normal. A p-value of <0.05 was considered statistically significant.

**Ethical clearance**

This research was declared ethically cleared by the Ethics Research Commission, Faculty of Medicine, Udayana University, under No. 2023.03.1.095.

## RESULTS

**Effect of *H. scabra* on parasitemia**

The parasitemia data in the control and treatment groups are presented descriptively in **Table 1**. The analysis of variance showed that the lowest percentage of parasitemia was found in the group given a dose of 100 mg/kg BW *H. scabra*, namely 9.4%, with a significant difference between the four groups (p=0.000). The multiple comparison test showed that the percentage of parasitemia was not significantly different between groups P1, P2 and P3 (p>0.05) (Table 2). Therefore it was concluded that 100 mg/kg of *H. scabra* is effective as schizontocidal and for malaria prophylaxis.

Table 1. The *H. scabra* simplicial activity on percentage of parasitemia and parasite inhibitory percentage of mice infected with *P. berghei*

| | P0 (n=6) | P1 (n=6) | P2 (n=6) | P3 (n=6) | p value |
|---|---|---|---|---|---|
| % parasitemia | 36.66±10.04 | 11.18±1.83 | 9.38±1.59 | 15.17±2.72 | 0.000 |
| % inhibition | ---- | 32.43±8.99 | 27.29±8.97 | 43.83±14.17 | 0.054 |

Note: Values are presented as mean ± SD

P0: control group given CMC 1%; P1 was given a dose of 10 mg/kg BW (human dose converted to mice) Peter's 4-day suppressive test method; P2 was given a dose of 100 mg/kg BW using the Peter's 4-day suppressive test method; P3 was given a dose of 100 mg/kgBW as prophylaxis before being infected with *Plasmodium berghei.*

**Effect of administering *H.scabra* on the percentage of parasite inhibition**

Descriptive data of the effect of *H. scabra* administration on the percentage of inhibition of parasite growth in mice infected with *P. berghei* is shown in **Table 1**. It was found that the highest percentage of growth inhibition of *P. berghei* was in group P3 that received 100 mg/kg BW *H. scabra*, namely 43.8%. However, there was a non-significant difference between the four groups (p=0.054).

Table 2. Multiple comparison test of percentage of parasitemia

| % of parasitemia | Mean difference | p value |
|---|---|---|
| P0 vs P1 | 25.48 | 0.000 |
| P2 | 27.28 | 0.000 |
| P3 | 21.49 | 0.000 |
| P1 vs P2 | 1.80 | 0.931 |
| P3 | 3.99 | 0.573 |
| P2 vs P3 | 5.79 | 0.261 |

Note: P0: control group given CMC 1%; P1 was given a dose of 10 mg/kg BW (human dose converted to mice) Peter's 4-day suppressive test method; P2 was given a dose of 100 mg/kg BW using the Peter's 4-day suppressive test method; P3 was given a dose of 100 mg/kgBW as prophylaxis before being infected with *Plasmodium berghei*.

## DISCUSSION

The results of this study indicate that *Holothuria scabra* at a dose of 100 mg/kg BW is most effective in decreasing the parasitemia in mice infected with *P. berghei*. Research on the cytotoxicity of sea cucumbers shows that only the methanol extract has an LC50 toxicity of 50.5µg/mL.[14] There are no research studies stating that extracts of *H. scabra* in other solvents have toxic effects and also there has never been any previous research examining the antimalarial activity of *H. scabra*. However, there are a large number of other in vitro research studies on the effect of other Holothuria spp., such as *H. atra*, on *Plasmodium*.[15]

Jattujan et al.[16] showed that *H.scabra* has antioxidant and antiaging biological activity with its bioactive content being triterpene glycosides. The mechanism of action of *H. scabra* simplicia as an antioxidant and an antiaging substance is through the insulin/IGF-1 (IIS) and DAF-16 signaling pathways. The nutritional content of sea cucumber viscera includes flavonoids, saponins, phenols, proteins, amino acids, lipids, fatty acids, and oligosaccharides.[17]

The biological and pharmacological activities of various sea cucumber species include antioxidant, anti-angiogenic, antitumor, anticancer, antimicrobial, anticoagulant, antithrombotic, antihypertensive, anti-inflammatory, and wound healing activities.[18,19]

The bioactive components of sea cucumbers that probably cause these biological activities are mainly triterpene glycosides (saponins), glycosaminoglycans (GAG), chondroitin sulfate, sulfated polysaccharides, phenols, cerebrosides, peptide lectins, glycosphingolipids, sterols (glycosides and sulfates), glycoproteins, and essential fatty acids.[18]

The bioactive compounds found in *Holothuria* include polysaccharides, triterpene glycosides or saponins, peptides, phospholipids, glycolipids, and phenols.[20]

Other research studies also showed that sea cucumbers have antifungal, cytotoxic, hemolytic, cytostatic, and immunomodulatory biological activity. The phenol content of *H. scabra* is quite large and has the highest antioxidant activity.[21] Eight of the core triterpenoid biosynthesis enzymes were discovered, but the identity of the specific saponin biosynthesis pathway enzymes remains unknown. It has been confirmed using solid phase extraction followed by ultra-high-pressure liquid chromatography-quadrupole time of flight-mass spectrometry that the body wall releases at least three different triterpenoid saponins. The resources that we have will help guide future research to explore the biosynthesis of secondary metabolites in sea cucumbers.[22]

*Holothuria scabra* is also known to have activities that suppress inflammation, promote wound healing, and increase immunity. Extracts from *H. scabra* also contain many bioactive

compounds that have a strong inhibitory effect on the survival and development of tumor cells.[23] It was also discovered in one previous study that the reproductive process of *H. scabra* is associated with sex steroids, therefore the identification of steroid biosynthetic genes from *H. scabra* may be possible in the future.[24]

Moelyadi et al.[25] using the molecular docking method showed that the cathechin content of *H.atra* has an inhibitory effect on the development of *P. falciparum*. *Holothuria scabra* extract also reduces reactive oxygen species, therefore *H. scabra* is a potential candidate for the prevention and natural therapy of Alzheimer's dementia.[26] Research by Utami et al.[27] found that *H. atra* is a potential antimalarial candidate in silico. The active ingredient which is thought to act as an antimalarial is a triterpene glycoside.[28] However, the mechanism of action of *H.scabra* as an antimalarial requires further investigation.

The limitation of this study is that no hematological parameter analysis was conducted. This study would be valuable for future research in developing a new antimalarial drug derived from natural resources. Further research is required to formulate extract preparations as well as qualitative and quantitative phytochemical tests for *H. scabra* to determine secondary metabolites or active ingredients that are thought to have an antimalarial role.

## CONCLUSIONS

Based on the results of this study that has been carried out, it can be concluded that using both the four day suppressive test and prophylaxis methods, *H. scabra* has antimalarial activity. *H. scabra* at a dose of 100 mg/kg BW was most effective in decreasing the percentage of parasitemia and tended to inhibit parasite growth in mice. Further research needs to be carried out to determine the mechanism of action of *H. scabra* as an antimalarial.

### Conflict of Interest

The authors declare no conflicts of interest.

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### Author Contributions

DAASL conceptualized and designed the study, collected data, drafted the initial manuscript, obtained the funding, and reviewed and revised the manuscript; PAAD and IMS collected data, drafted the initial manuscript reviewed and revised the manuscript, IKT and LPRS conducted the initial analyses, drafted the initial manuscript, PIBA and NLR collected data, reviewed and revised the manuscript; and all authors have read and approved the final manuscript as submitted and agree to be accountable for all aspects of the work. All authors have read and approved the final manuscript.

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### Data Availability Statement

The data that support the findings of this study are available on request from the corresponding author.

### Declaration of use of AI in Scientific Writing

We declare that we do not use AI in our scientific writing.

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