



Original Article

Comparing Black Trepong and Curryfish Extract's Antimalarial Activity Using *In Vitro* Screening

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ABSTRACT

Background: Malaria resistance to artemisinin combination therapy has prompted researchers to explore anti-plasmodium materials. One of the marine biotas thought to have the potential to inhibit the development of malaria parasites (*Plasmodium sp*) are black trepong and curryfish. These two marine biotas consist of two varieties of sea cucumbers with various therapeutic benefits. However, their antimalarial effects have not been studied.

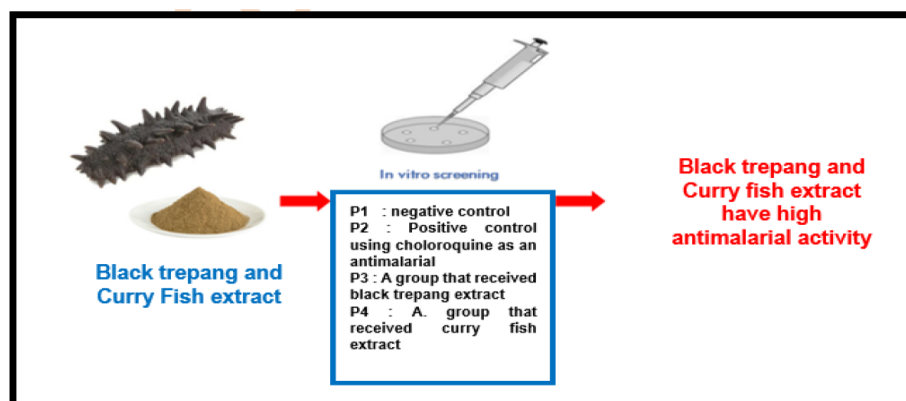
Objective: Two marine biotas, black trepong and curryfish extract, were analyzed using *in vitro* screening to determine their antimalarial activity.

Methods: The experiment was done through *in vitro* screening technique (culture medium containing *P. falciparum*). P1 was a negative control; P2 was a positive control/using chloroquine as an antimalarial; P3 was a group that received black trepong extract) and P4 was a group that received curryfish extract. Measurements of parasitemia levels, growth percentage, inhibition rate, and IC₅₀ were performed to determine the antimalarial activity.

Results: The antimalarial results suggest that increasing the dose of marine biota extract reduces the parasite development rate and increases the inhibition rate. In line with IC₅₀ analysis, both marine biotas have high antimalarial activity. Curryfish extract has a lower IC₅₀ value than black trepong extract.

Conclusion: The study demonstrated that black trepong and curry fish have high antimalarial activity.

GRAPHICAL ABSTRACT



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Introduction

Malaria is a global epidemic that can be lethal, especially for high-risk individuals including babies, toddlers, and expectant mothers. Likewise, malaria contributes directly to anemia and can decrease labour performance. Malaria eradication has resulted in a decrease in morbidity and mortality [1]. Still, malaria control efforts over the last decade have been ineffective [2]. Malaria control is made worse by the COVID-19 pandemic, which affects the decline and slowdown of care for this infectious disease. This condition increased morbidity rates of 240 million cases and a mortality rate of around 627 thousand [2, 3].

During the last 20 years, artemisinin combination treatment, or ACT, has been the main medication for treating various malaria infections. Nevertheless, cases of ACT resistance have been reported in the Southeast Asian region [4]. Even the most recent reports indicate that ACT resistance has occurred on the African continent in Rwanda [5] and Northern Uganda [6]. Malaria eradication efforts are in jeopardy if ACT activity declines. Finding novel therapeutic strategies has become vitally critical in the fight against the emergence of ACT-resistant parasites.

The waters comprise 2/3 of the planet's surface and are habitat to diverse aquatic species. Numerous active substances with therapeutic effects are present in marine life. A significant increase in phylogenetic diversity is among the benefits of marine biota over land biota. The phylogenetic diversity is brought by various environmental stimulations, necessitating various adaptations and enabling the marine biota to produce unique active compounds with therapeutic value [7].

Curryfish (Latin name: *Sticophus hermanni*) is a type of sea cucumber found in shallow water and coral reefs [8]. Little black or dark brown papillae strewn across both the lateral and dorsal sides, as well as its light yellow or green yellow coloring, are its distinguishing features [9]. Curryfish has several active compounds such as glycosaminoglycans, keratin, glucosamine, triterpene glycoside, carotenoids, peptides, fatty acids, a natural antiseptic, minerals, omega 3 and

6, lectins, mucopolysaccharides, glycosides, chondroitin, cell growth factors, and collagen [10]. According to the prior studies, curryfish's phytochemical assay contains various active molecules including terpenoid, tannin, steroid, saponin, and also flavonoid [11]. The black trepang or brown trepang (*Holothuria atra*) is a reddish black sea cucumber with small and dense long papillae covering its dorsal surface. These sea cucumbers are found in all shallow waters of Indonesia, especially near sandy beach areas and coral reefs [12]. Previous research has shown that black trepang phytochemical analysis contains various active compounds, including alkaloid, flavonoid, phenol hydroquinone, glycosides, saponins, steroid, and triterpenoids [13, 14]. Several active ingredients include triterpenoids [14], alkaloids, flavonoids, phenol, and saponin have antimicrobial properties [15]. Black trepang and curryfish become part of the community's nutrition and have been shown to contain various active substances with antifungal and antibacterial properties [16, 17]. Previously, the ability of *Holothuria atra* or black trepang to act as an antimalarial was investigated using an *in silico* method against the *P. falciparum* orotidine 5-monophosphate decarboxylase (PfOMPDC) protein [18]. Research conducted *in silico* on curryfish's antimalarial properties further revealed that it inhibits glucose metabolism via *Plasmodium falciparum* hexose transporter (PfHT1) [19]. However, no previous *in vitro* studies have been conducted comparing the antimalarial activity of the two species. The phenomenon of ACT resistance, increased malaria mortality-morbidity, bioactive components in sea cucumbers, and no *in vitro* study has been conducted to compare the antimalarial activity of two marine cucumber species, initiated research on the antimalarial properties of extracts from black trepang and curryfish. Utilizing marine biotas, such as black trepang and curryfish can be used as an alternative treatment to counter malaria drug resistance. This study evaluated curryfish and black trepang extracts' antimalarial potency against *P.falciparum*, using *in vitro* approach. Sea cucumber research and development should be

continued to produce antimalarials that are effective but have few side effects.

Materials and Methods

Research design

The research design was a true experimental study that compared the experimental group and control group and observed the possibility of causation between both groups. The true experimental research methodology employed is a posttest-only control group design, in which the impact of treatment administration is measured at the end of the research. The research sample was *P. falciparum* culture medium. Each medium had the same amount of parasitemia, and the medium sample was distributed randomly among the research groups. The total number sample of this research was 40 sample mediums, which were divided into four groups:

P1: Negative control group, *P.falciparum* medium without administration of medicine or any extract.

P2: Positive control group, *P.falciparum* medium with antimalarial drug (chloroquine) administration.

P3: *P.falciparum* medium with the administration of black trepang extract.

P4: *P.falciparum* medium with the administration of curryfish extract.

P2-P4 groups will be given graded doses extract/drug, including 0.01, 0.1, 1, 10, and 100 µg/ml [20]. Each type of dose will be administered to two culture medium samples in each group. The treatment description in this study can be seen in the following scheme (Figure 1):

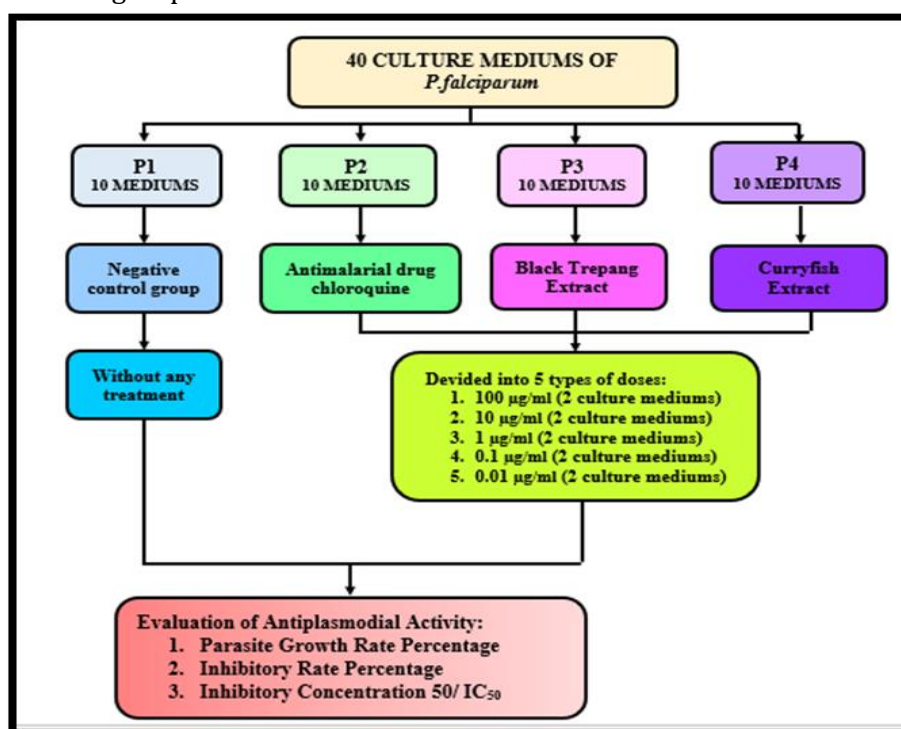


Figure 1: Research scheme

$$\text{Parasite growth (\%)} = \frac{\text{Parasitemia level following treatment} - \text{Parasitemia level before treatment}}{\text{Parasitemia level before treatment}} \times 100$$

$$\text{Inhibition Rate (\%)} = 100 - \left[\frac{\% \text{ Plasmodium growth in treatment group (P2-P4)}}{\% \text{ Plasmodium growth in negative control group (P1)}} \times 100 \right]$$

Figure 2: Formula to determine parasite growth and inhibition rate [25]

The Ethics Committee of Hang Tuah Medical Faculty has given its official approval for the research's implementation. All phases of this research, including materials and *in vitro* testing, were carried out at Airlangga University's Malaria Laboratory - Tropical Disease Institute.

Black trepang and curryfish material

During the rainy season, the marine materials used in this study (black trepang and curryfish) were collected from the sea area near Sapeken Island, Sumenep, Madura-East Java, Indonesia. Taxonomic tests were performed on fresh black trepang and curryfish at the Plant Bioscience and Technology Laboratory, Institut Teknologi Sepuluh November, Surabaya, Indonesia.

Extraction technique

The maceration or cold extraction technique is advantageous for this study in several ways. The inexpensive cost, more active components dissolve, a lot of extracts are produced, and the technique does not require heating, which lowers the chance that the ingredients will be broken down [21, 22].

The extraction process began with removing all internal organs from the meat and dried in a -50 °C freeze dryer. After drying, the meat of black trepang and curryfish is crushed into a powder (1 kg of wet black trepang and curryfish yield approximately 900 grams of powder). The sea cucumber powder was macerated with n-hexane solvent three times for twenty-four hours at room temperature, and then filtered through filter paper. The raw extract is obtained by rotating evaporating the resultant macerate at 60 °C. The raw extracts were kept refrigerated until needed in an airtight container [21, 22].

In vitro formation of *plasmodium falciparum*

Three fundamental components must be used for the culture technique: (1) Human blood plasma and packed red blood cells provided by the Red Cross, (2) Strain of *P.falciparum*, which is sensitive to chloroquine, and (3) medium RPMI 1640. This study conducted an *in vitro* experiment using a modified Trager and Jensen methodology [23]. The first stage was initiated by

dissolving 50 g/mL hypoxanthine, 5.94 g HEPES, and clean water in a beaker glass and stirring until homogeneous. To make 1 liter of solution, 10.4 g of RPMI-1640 or Roswell Park Memorial Institute 1640 media and clean water were added. The mixture was then supplemented with 2 mg/mL sodium bicarbonate (NaHCO₃), 5.94 g/L HEPES or N-2-hydroxyethyl piperazine-N-2-ethane sulfonic acid, 25 mg gentamicin, and 10% serum from human blood [24, 25].

The screening of antimalarial activity

500 µl of parasite cell suspension (with 1% parasitemia level and 5% hematocrit) was placed into a 24-well microplate. With 5% sorbitol, the parasites remained kept in their ring-stage phase. 10 mg of chloroquine diphosphate (used as a positive control) and of black trepang - curryfish extracts were diluted in 1 mL of the solvent dimethyl sulfoxide or DMSO, which was then serially diluted using RPMI-1640 to make several concentrations (0.01, 0.1, 1, 10, and 100 g/mL) [24, 25]. DMSO was further employed as the negative control. The following step is to incubate the parasite culture media in a candle jar for two days at 37 °C, 5% CO₂, 5 % O₂, 90 % N₂, and 95% humidity [26]. The density of *P. falciparum* parasites was observed three times (n=3) by microscopic examination of thin peripheral blood smear stained with Giemsa and treated with methanol. A light microscope was used to examine the infected erythrocytes at 1000-fold magnification. The homogenized ring-stage *P. falciparum* parasites was used with a parasitemia density of about 1 percent [25, 27]. The parasitemia levels were rechecked after the two-day incubation period to assess the growth rate and degree of inhibition rate. This dataset was used to evaluate the parasitemia growth and inhibition rate (Figure 2).

The third step to evaluate the antimalarial activity of the extract was IC₅₀. Inhibitory Concentration 50 or IC₅₀ is a method for assessing the potency of a drug or active ingredient. In pharmacological research, the IC₅₀ number calculates the drug/active ingredient required to reduce living organisms to 50%. In addition,

Probit statistical analysis was used to calculate the IC₅₀ [28, 29].

Antimalarial activity assessment standard

Based on their IC₅₀ values, the extract substances were divided into four groups and evaluated by comparison to the WHO guidance and standard criteria for antimalarial drug research activities (Figure 3).

Research data analysis

The research analysis is divided into two stages:

- (1) Importing all datasets into Excel, including parasitemia level, parasite growth percentage, and parasite inhibition percentage, and
- (2) Entering all relevant data, such as parasitemia level, parasite growth percentage, and parasite inhibition percentage, into SPSS for probit regression analysis to determine the IC₅₀. The probit analysis in this study assesses the drug concentration of black trepang and curryfish extract required to inhibit 50% of parasites [28, 29].

Results and Discussion

Evaluation of parasite growth percentage

The data below shows the results of the parasite growth after 48 hours of incubation (Table 1). All groups had homogeneous levels of parasitemia before incubation (parasitemia levels in all groups were 1.03%). The parasite growth (%) calculated by subtraction of parasitemia levels following black trepang and curryfish extract administration as well as parasitemia levels before the extract administration.

According to the statistics presented in Table 1, the mean result of the percentage of parasite

growth indicates that a decrease will follow the increase in the chloroquine dose and extract in parasite growth. The parasite growth rate at P2 (positive control) in all quantities was lower than in the group receiving black trepang and curryfish extracts. In P2, it was not necessary to measure the parasite growth at a dose of chloroquine of 100 µg/mL because the development at a dose lower than it (dose 10 µg/ml) showed a very low growth rate, making it unnecessary to measure again at the subsequent dose. The percentage of parasite growth at P4 was lower than in P3. Furthermore, the percentage of parasite growth at P3 was lower at 10 g/mL extract than at P4.

Evaluation of inhibition rate percentage and IC₅₀

An active ingredient's ability to stop an organism's growth is called inhibitory rate percentage. The formula for calculating the inhibitory rate can be seen in (Figure 2). The probit regression analysis method calculated the IC₅₀ based on parasite growth and inhibitory rate information. The information in (Table 2) indicates the results of measuring the inhibitory rate (%) and inhibitory concentration 50 (IC₅₀). As it can be seen in (Table 2), an increase in the drug's dose/ extract seems to increase the inhibitory rate. At 100 µg/mL extract doses, the maximum inhibitory rates were seen at P3 and P4. No calculation was made for the P2 inhibitory rate at a 100 µg/mL drug dose because the growth rate data was also not computed. Since no drugs or extracts were administered in P1, no data on calculating the inhibitory rate was available. The inhibitory rate at P4 was higher than at P3 (extract's dose 0.01 until 10 µg/mL).

- 1) If IC₅₀ ≤ 1 µg/ml are considered very high potency
- 2) If the IC₅₀ ≤ 5 µg/ml, it is considered high potency.
- 3) If the IC₅₀ is > 5 µg/ml or ≤ 15 µg/ml, it is considered encouraging potency.
- 4) If the IC₅₀ is > 15 µg/ml or ≤ 50 µg/ml, it is considered moderate potency.
- 5) If > 50 µg/ml is considered low potency.

Figure 3: The WHO guidance criteria for antimalarial drug research [25]

Table 1: Evaluation of average parasite growth percentage

Type of Extract/ Chloroquine Dose	Average of Parasite Growth (%) After Extract Administration			
	P1	P2	P3	P4
1	5.68	2.62	5.25	5.10
2	5.68	1.75	4.11	4.07
3	5.68	1.07	2.88	2.95
4	5.68	0.44	1.70	1.62
5	5.68	-	0	0

NB: 1: 0.01 µg/ml, 2: 0.1 µg/ml, 3: 1 µg/ml, 4: 10 µg/ml, and 5: 100 µg/ml.

P1 = control (-) group, P2 = control (+) group, P3 = black trepang group, and P4 = curryfish group.

Table 2: Evaluation of inhibitory rate percentage and IC₅₀

Type of Extract/ Chloroquine Dose	Average of Inhibitory Rate (%) after Extract Administration			
	P1	P2	P3	P4
1	-	53.50	7.69	10.49
2	-	69.06	27.45	28.50
3	-	81.64	48.78	49.65
4	-	92.31	69.58	71.85
5	-	-	100	100
IC ₅₀	-	0.005	1.23	1.07

NB: 1: 0.01 µg/ml, 2: 0.1 µg/ml, 3: 1 µg/ml, 4: 10 µg/ml, and 5: 100 µg/ml.

P1 = control (-) group, P2 = control (+) group, P3 = black trepang extract group, and P4 = curryfish extract group.

IC₅₀: Inhibitory concentration 50.

However, the data revealed that both groups' inhibitory rates were the same at 100 µg/mL extracts. The IC₅₀ data shows the dose of active ingredient that can suppress parasite growth by 50%, so when the IC₅₀ number is lower, the antimalarial activity increases. P2 has the lowest IC₅₀, followed by P4, and finally P3.

The research data revealed high antimalarial activity in black trepang and curryfish extract, based on parasite growth parameters, inhibitory rate, and IC₅₀ measurement. According to the WHO guidelines for pharmacological research, a substance if its IC₅₀ value falls below or equal to 5 µg/ml, it is classified as a strong antimalarial [25]. Black trepang's IC₅₀ (1.23 µg/mL) was higher than curryfish's IC₅₀ (1.07 µg/mL), which means curryfish has higher potency in reducing parasite development than black trepang. This research was an *in vitro* study. Therefore, all external and internal elements may be controlled, resulting in uniform conditions in each medium with a limited response to infection. The effect of administering the extract is more visible under homogenous research conditions and narrow responses. Adding the extract dose will increase

the extract's working activity. The activity improvement was probably due to an increase in active substance concentration, which is in line with increased extract dosages. That is, when an extract's active component content is higher, its antimalarial activity increases. There is a difference in the ability to impede rate between the two marine biotas due to variations in the composition and concentration of bioactive substances, which affect the antimalarial efficacy. Different IC₅₀ values were found for the two marine biotas in this study. Still, because both had IC₅₀ values were less than 5 µg/mL, they had the potent antimalarial activity.

Chloroquine, on the other hand, has a lower IC₅₀ than the other two marine biotas. The P2 (positive control group that received chloroquine as antimalarial) had the lowest IC₅₀ value in this study, indicating that chloroquine has the most significant action. Chloroquine's highest activity was possible because the strain of *P. falciparum* used in this experiment was chloroquine susceptible.

The anti-plasmodial activity of black trepang and curryfish is associated with various active

ingredients such as flavonoids, triterpenoids, alkaloids, saponins, and tannin. These active ingredients have antimicrobial properties, which can reduce parasite growth through many pathways [13-15]. An earlier study demonstrated that the flavonoid components interact strongly with the falcipain-2 receptors. Falcipain-2 was a cysteine proteolytic enzyme identified in the *Plasmodium* feeding vacuole and plays a significant role in the hemoglobin degradation pathway. At the erythrocytic stage of *P. falciparum*, hemoglobin had to be degraded to produce amino acids. Inhibition of falcipain-2 function causes the accumulation of non-degradable hemoglobin in food vacuoles [25]. *In vivo* study found that flavonoids are easily transformed into radical anions under a cellular oxidative stress environment, which severely induces the destruction of proteins, RNA, and DNA [30].

Several *in vitro* studies found derivatives of triterpenoids to have antimalarial activity. Unfortunately, the precise mechanism of action is currently unexplained. The possible mechanism of triterpenoids as antimalarial action includes their impacts on mitochondrial membrane potential and the reduction of β -haematin synthesis [31-33]. Triterpenoid (betulinic acid) antimalarial activity is assumed to be related to its ability to engage the PfATP6 protein, which regulates calcium ion transportation and affects the host-parasite selectivity [34].

Several alkaloid compounds have been demonstrated *in vitro* to have significant antimalarial action. A low IC_{50} value indicates that the antimalarial activity is significant [35]. However, some derivatives are highly toxic, while others are unknown in terms of toxicity. Alkaloids' mechanisms of action have also not been investigated, so more research is required to determine the alkaloids pathway [37, 38]. The experimental studies in mice have demonstrated the antimalarial activity of saponin derivatives by inducing oxidative stress, which causes cell and tissue damage [39, 40]. According to review studies, saponin derivatives can act as antimalarial with low toxicity. Saponins derivatives may inhibit parasite development through three mechanisms, including (1) induced

disorganization of membrane lipid raft, (2) inhibition of HMGB1 (high mobility group box 1) is a protein that affects the transcription process, and (3) potential inhibition of the detoxifying enzyme glyoxalase [41]. Tannins are bioactive substances with antimalarial properties and an unknown action mechanism [42]. However, tannins may have the ability to induce the crystallization of hemozoin pigment (a byproduct of hemoglobin metabolism) as well as inhibit protein biosynthesis and nucleic acid fragmentation [42, 43].

According to the findings of this study, all the active bioactive compounds should be determined using liquid chromatography-mass spectrometry (LCMS) on both marine biotas. We should do *in vivo* studies involving animal models to ascertain the antimalarial activity, the most effective preparations and solvents for the extraction process, the mode of action of the extracts, and the extract's effects on other tissue, such as the liver, spleen, and kidneys. A thorough investigation of the antimalarial activity of black trepang and curryfish is expected to be a breakthrough in discovering a new regimen for malaria based on a natural product and the minimum side effects.

Conclusion

The outcomes of this study show that black trepang and curryfish have significant antimalarial efficacy. Although the IC_{50} of curryfish is smaller than that of black trepang, both fulfill the WHO standards as active substances with high antimalarial potential. More research into curryfish and black trepang can be done by employing malaria mice models to ascertain their antimalarial action, immunological regulators, and reduced oxidative stress.

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Authors' Contributions

All authors contributed to data analysis, drafting, and revising of the paper and agreed to be responsible for all the aspects of this work.

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