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Evaluation of Antimalaria Activity and Acute Toxicity of *Xetospongia* sp

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ABSTRACT

We investigated the *in vivo* activity of extract of *Xetospongia* sp against *Plasmodium berghei* Strain ANKA and acute toxicity in mice. The ethanolic extracts of the *Xetospongia* sp (50-400 mg kg^{-1} day) were screened for blood schizonticidal against *P. berghei* strain ANKA in mice during early and established infections. Acute toxicity of extracts of *Xetospongia* sp at the dose 5000 mg kg^{-1} on mice administeredorally exhibited no toxicity, as evident in that fact that no histopathological changes were observed in some essential organs, such as liver, heart, digestive tract and kidney. The ethanolic extracts (50-400 mg kg day^{-1}) exhibited a significant antimalarial activity both in the 4-day early infection test and in the established infection and they were found to be relatively safe up to a dosage of 5000 mg kg^{-1}, with an LD_{50} value of >500 mg kg^{-1}.

Keywords: *Xetospongia* sp, Antimalarial Activity, Acute Toxicity

1. INTRODUCTION

Malaria is a disease of the tropics and subtropics. It is a global problem, endemic in 106 countries with an estimated 3.3 billion people at risk of malaria in 2010. In 2010 there were 216 million cases of malaria world wide, with an estimated 655,000 deaths WHO, 2011. In Indonesia, 1.25 to 2.5 million probable malaria cases with 45 to 50% being cases of *Plasmodium falciparum*, nearly 350,000 confirmed malaria cases and around 500 confirmed malaria deaths, are reported every year (SEARO, 2011).

The rise of antimalarial drug resistance has dominated global malaria control programs since resistance to chloroquine was first documented in patients in 1959. Chloroquine was the first widely used modern antimalarial, but within little more than a decade after its introduction Chloroquine Resistant (CQR) *P. falciparum* had emerged and begun to spread across Asia. In areas of high grade CQR a new drug; Fansidar (Sulfadoxine-Pyrimethamine, SP) was adopted as the recommended therapy for uncomplicated falciparum malaria (Sibley and Price, 2012).

Drug resistance accompanied by lack of progress in the development of vaccines or resistant reversal agents has further aggravated the situation. Marine derived compounds have been explored and considered as possible anti-malarial agents. The marine sponge, *Axinyssa djiferi*, collected on mangrove tree roots in Senegal, was investigated for glycolipids have potent antimalarial activity (Farokhi *et al*., 2013). A new bispyrroloiminoquinone alkaloid, tsitsikammamine C, from the Australian marine sponge *Zyzzya sp* displayed potent *in vitro* antimalarial activity and inhibited both ring and trophozoite stages of the malaria parasite life cycle (Davis *et al*., 2012).

One compound of sponge that has been found to have antimalaria activity is manzamine A. Manzamine A, a b-

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carboline alkaloid, was shown to inhibit the growth of the rodent malaria parasite *Plasmodium berghei*, not only *in vitro* but also *in vivo* (Ang *et al*., 2000). In the last two decades, the manzamine alkaloids have represented an important class of natural products for the development of new bioactive molecules for the treatment of malaria. Manzamin A alkaloids can be isolated from the sponge family Petrosiidae (Samoylenko *et al*., 2009).

Sponge from the genus of Petrosia commmonly found in Situbondo waters, East Java, Indonesia is *Xetospongia* sp (Abdillah *et al*., 2013). *In vivo* antimalaria test and acute toxicity test against *Xetospongia* sp were conducted as an effort to find bioactive compounds from natural ingredients, particularly sponge.

2. MATERIALS AND METHODS

2.1. Animal Materials

Sponge samples of *Xetospongia* sp was collected by scuba diving at a depth of about 8 and 500 m from the coastline in Tajung Pecaron Bay, near Situbondo, Indonesia. The selected sponge, *Xetospongia* sp. was well identified with the help of Laboratorium of Ecology of Marine, Department of Biology, Institute of Technologi Surabaya. A voucher specimen (No A24354) was deposited at Department of Biology, Faculty of Sciences, Institute Technology of Surabaya. The strain of *P. berghei* was provided by the Centre of Bioscience and Biotechnology, Faculty of Sciences and Technology, National University of Malaysia.

2.2. Extract Preparation

Freeze-dried or wet samples of the sponges were soaked twice in ethanol. Each soaking lasted for 24 h. After filtration, the ethanol extracts were then evaporated under reduced pressure in a rotary evaporator and evaluated for their effectiveness against *P. berghei* parasites.

2.3. Parasite Inoculation

A single donor mouse infected with *Plasmodium berghei* parasites was blood into sterile heparinized culture medium and the blood was diluted with RPMI 1640 medium. The healthy experimental mice were infected intravenously via a tail vein with 0.2 mL of the diluted blood containing 1×10^7 parasitized (*Plasmodium berghei*) red blood cells on day one.

2.4. Evaluation of Antimalarial Activity of the Extract of *Xetospongia* sp

Evaluation of suppressive activity of the extract (4-day test).

This test was used to evaluate the schizontocidal activity of the extract and chloroquine against early *P.berghei* infection in mice. This was done as described by Knight and Peters (1980) on the first day (Do), the seventy-two mice were infected with the parasite and randomly divided into various groups (n = 6). These were administered with the extract and chloroquine. The mice in group 1 were administered with 400 mg kg^{-1}, group 2, 200 mg kg^{-1}, group 3, 100 mg kg^{-1}, group 4, 50 mg kg^{-1} of the crude extract, Chloroquine was given to the positive control group (group 5) and 10 mL kg^{-1} of distilled waterto negative control group (Group 6) for fourconsecutive days (D0-D3) between 8am and 9am. On thefifth day (D4), thin blood film was made from tail blood.The film was then stained with Leishman's stain to reveal parasitized erythocytes out of 200 in a random field of themicroscope.

2.5. Evaluation of Curative Activity (Rane's test)

This was used to evaluate the schizontocidal activities ofthe extracts and chloroquine in established infection. Thiswas done as described by Ryley and Peters (1970). *P.berghei* was injected intraperitoneally into groups of mice (n = 6) on the first Day (Do). Seventy-two hours later (D3), the mice was divided randomly into groups of six mice each. Different doses of the leaf extract, 400, 200, 100 and 50 mg kg^{-1} were orally administered respectively to mice in groups 1-4, the extract, 5 mg kg day^{-1} of chloroquine (positive control) and 10 mL kg^{-1} of distilled water (negative control) were respectively given to mice in Groups 5 and 6. The extract and drugs were administered once daily for5 days. Leishman's stained thin smears were prepared from tail blood samples collected on each day of treatment to monitor parasitaemia level. The Mean Survival Time (MST) of the mice in each treatment group was determined over a period of 29 days (D0-D28).

2.6. Acute Toxicity Test

The acute toxicity of extracts weretested on rats using 3 doses (625, 2500 and 5000 mg kg^{-1} body weight) administered orally. Control rats were kept under the same conditions without any treatments. The animals were routinely inspected for appearances or signs of toxicity such as tremors,weakness and refusal of feeds, falling off hair, coma or even death for 48 h. The Lethal Dose LD_{50} was estimated from the graph of percentage mortality converted to probit against log-dose of the extract, probit 5 being 50%.

3. RESULTS

3.1. Evaluation of Suppressive Activity of the Extract (4-Day Test)

On the day 5, extract of *Xetospongia* sp yielded %suppression of 60,23% for a dosage of 400 mg kg day^{-1}, 42,48% for a dosage of 200 mg kg day^{-1}, 25,91% for a dosage of 100 mg kg day^{-1} and 3,86% for a dosage of 50/kg/day. Based on these findings, treatment groups receiving extracts at a dosage of 400 mg kg day^{-1} exhibited a lower parasitemia rate than any other treatment groups even though it was still lower than that of positive control group (chloroquine treatment group).

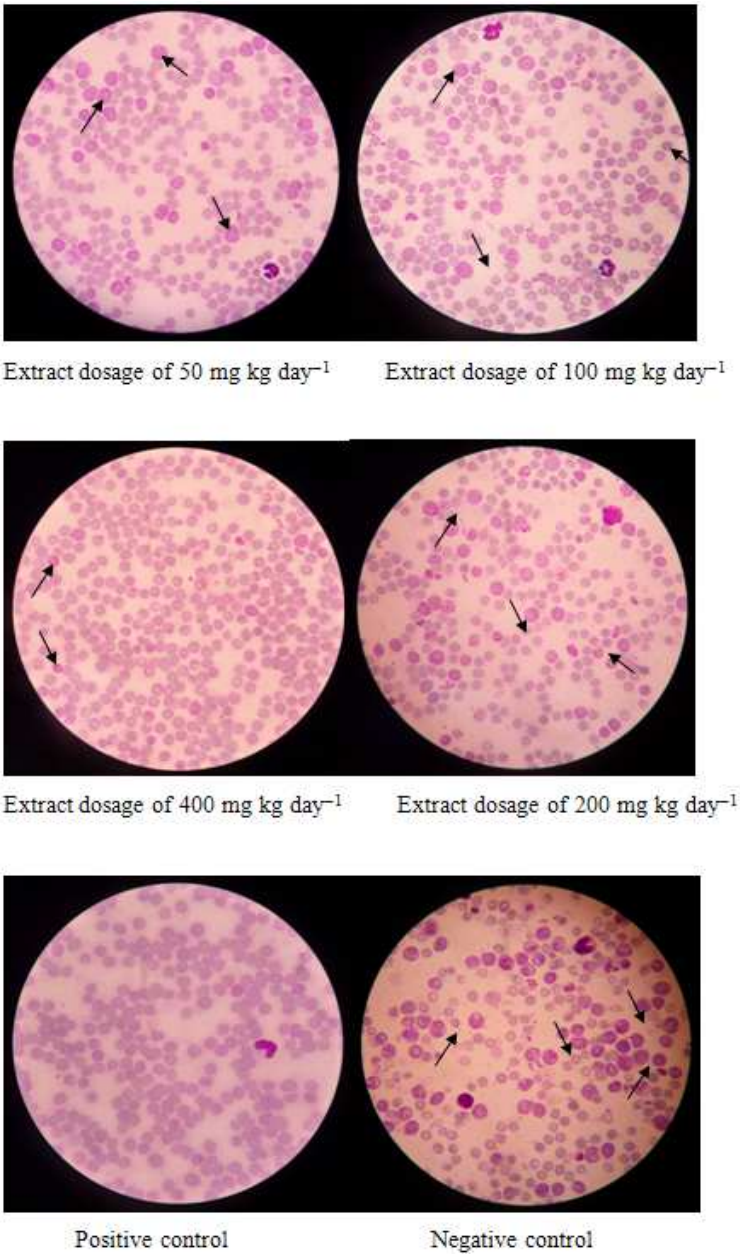

Fig. 1. Parasitemia density (thin blood smear) in combination test on the day 4, visualized using a light microscope (* arrow indicates parasite-infected erythrocytes)

Parasitemia density in each treatment group is depicted in **Fig. 1**. The best parasite inhibitory activity was exhibited by control positive group and treatment group receiving 400 mg kg day^{-1}.

In the positive control group, recovery of the experimental animal began on the Day 3 and on the Day 4, all mice were found to be negative in parasitemia test.

Based on percent inhibition exhibited by each test group, statistic analysis was conducted to determine the significance in the different of percent inhibitions in the treatment groups receiving the extracts and the control group receiving chloroquine. Kruskal-Wallis statistic analysis exhibited a significance value of p = 0.021. It means that at least three groups of mice had a significant difference in the percent inhibition ($p<0.05$).

3.2. Acute Toxicity Test

Acute toxicity tests were done for *Xetospongia* sp. Three groups of mice, eachgroup having 5 male and 5 female mice, for dose levels (625, 2500 and 5000 mg kg^{-1}) were used for toxicity test. The mice were acclimatized and fasted over night. The weight of each mouse was measured and calculated for all the dose levels. The extracts were given to the mice before they ate anything (empty stomachs). The test was repeated with doses (625, 2500 and 5000 mg kg^{-1}) orally. Toxicity signs such as death, changes in physical appearance, behavioral change and organ damage were observed for 48 h.

The table above shows that up to a dosage of 5000 mg kg day^{-1}, extracts of *Xetospongia* sp administered for 48 h did not lead to mouse death, with an LD_{50} value of >5000 mg kg^{-1}.

4. DISCUSSION

Marine sponges have a potential to provide future drugs against important diseases, such as malaria, cancer and a range of viral diseases (Bhimba *et al*., 2013). Of 10,000 marine sponges, 11 genera are known to produce bioactive compounds and only three genera (*Haliclona*, *Petrosia* and *Discodemia*) are known to produce anti-malarial. *Xetospongia* sp is one of the sponges of genus Petrosia that are widely found in Indonesian water areas. *Xetospongia* is a genus of sponge that contains manzamine compound. Manzamines are a structurally unique group of b-carboline alkaloids isolated from a *Halliclona sp* but subsequently found in other genera of marine sponges including *Xetospongia* (Guzman *et al*., 2011). Ang *et al*. (2000) isolated manzamine-A, a, β carboline alkaloid in several marine sponges, inhibiting the growth of the rodent malaria parasite *Plasmodiumberghei in vivo*. They also suggested the manzamine- A and 8-hydroxymanzamine-A, as promising new antimalarial agents/technical status of different methods to produce sponge.

The *P. berghei*-infected mouse model has been widely used as a preliminary test for the *in vivo* activity of potential antimalarial agents, as it provides a preclinical indication of any *in vivo* potential bioactivity as well as possible toxicity of the sample tested.

4.1. Evaluation of Suppressive Activity of the Extract (4-Day Test)

The 4-day suppressive test is a standard test commonly used for antimalarial screening and the determination of percent suppression of parasitemia is the most reliable parameter (Gitua *et al*., 2012). Evaluation of antimalaria activity was based on %parasitemia 4 days after the test animals were given antimalaria treatment for 4 consecutive days. *Plasmodium berghei* is widely used for *in vivo* antiplasmodial activity assay since it is molecularly analogous to *P. falciparum*. By using *P. berghei* as a test *Plasmodium*, the research findings expectedly described the effects against *P. falciparum* as a *Plasmodium* that most commonly infects human being (Cox, 1988).

Malaria parasite infected mice exhibited the symptoms of trembling, anemia, paleness, weakness and reduced body weight. The mice looked pale due to anemia. The symptoms of anemia occurred since many erythrocytes were lysed due to parasite infection. Consequently, the counts of erythrocytes decreased. Reduced erythrocyte counts were evident from the fact that the mice blood was more diluted and pale. The mice also lost appetite for food. This led to decreasing body weight and weakness. The symptoms were similar to what human beings experience in case of malaria parasite infection. The

development of malaria is also determined by the immune system of the test animals and their response to the treatment.

Table 1 shows that administration of *Xetospongia* sp extract at a dosage of 200 mg kg day^{-1} was able to suppress parasite growth by 42,48%, while a dosage of 400 mg kg day^{-1} was able to suppress parasite growth by 60,23%. Therefore, the total ED_{50} value for the extract of *Xetospongia* sp administered on oral route was 331,95 mg kg^{-1}.

4.2. Evaluation of Curative Activity (Rane's test)

In the curative study, the ethanolic extract of *Xetospongia* sp produced a dose dependent reduction in parasitaemia levels in the extract treated groups; there was also a similar reduction in the chloroquine treated group (positive control). This finding is sufficient to say that *Xetospongia* sp extract has therapeutic efficacy against established malaria parasite.

The extract produced significant ($p \leq 0.05$) and dose- dependent decrease in parasite counts. The mean percentage of parasitaemia were 8,52, 9,49, 6,02 and 4,17 at the doses of 50, 100, 200 and 400 mg extract/kg body weight respectively **Table 2**. At 10 mg kg^{-1}, Chloroquine produced 98.42% chemo suppression. The extract produced significant ($p<0.05$) dose-dependently prolonged the survival time of mice while, chloroquine at 10 mg kg^{-1} body weight significantly ($p<0.001$) prolonged the survival time of treated mice.

4.3. Acute Toxicity Test

In general, if the Lethal Dose (LD50) of the test substance is three times more than the Minimum Effective Dose (MED), the substance is considered a good candidate for further studies. It was also suggested that oral administration is about 100 times less toxic than intraperitoneal (Prohp and Onoagbe, 2012). However, the hydro alcoholic extracts showed no lethality to mice at 5,000 mg kg^{-1}, which is 25 times the MED and no gross behavioral and physical changes were revealed (**Table 3**). Therefore, observations that no death with up to an oral dose of 5000 mg kg^{-1} could indicate that the test extracts are very safe.

Table 1. Effect of ethanolic extract of *Xetospongia* sp on suppressive activity

Extract/drug	Percentage of parasitemia	Percentage of suppressive
Xetospongia sp		
Dose 400 mg kg day^{-1}	3,07±0,98	60,23
200 mg kg day^{-1}	4,44±0,87	42,48
100 mg kg day^{-1}	5,72±0,96	25,91
50 mg kg day^{-1}	6,65±0,94	3,86
Vehicle	7,72±0,84	0
Chloroquine 10 mg kg^{-1}	0,13±0,97	98,32

Each results is mean of mice ± controls

Table 2. Antiplasmodial activity of the ethanol extract of *Xetospongia* sp. during established infection (Rane test)

Extract/drug *Xetospongia* sp	Percentage of parasitemia	Mean survival time (day)
Dose 400 mg kg day^{-1}	4,17±1,27	16,52±1,03*
200 mg kg day^{-1}	6,02±1,13	14,63±1,53*
100 mg kg day^{-1}	9,49±1,21	10,42±1,42*
50 mg kg day^{-1}	8,52±0,98	5,42±1,03
Chloroquine 10 mg kg^{-1}	0,00±0,09	30,0±0,00*

Data are expressed as mean ± SEM for five mice/group □ *p<0,05. When compared to control

Table 3. Physical changes of the ethanol extract of *Xetospongia* sp during acute toxicity test

Extract of *Xetospongia* sp	12 h later	24 h later	48 h later
Dose 625 mg kg^{-1}	No change behavior salivation	No change behavior	No change behaviour
2500 mg kg^{-1}	No change behavior salivation	No change behavior	No change behaviour
5000 mg kg^{-1}	Piloerection Rapid respiration salivation	No change behavior	No change behaviour

5. CONCLUSION

The ethanolic extract of *Xetospongia* sp produced a dose dependent reduction in parasitaemia levels in the extract treated groups; there was also a similar reduction in the chloroquine treated group (positive control). This finding is sufficient to say that *Xetospongia* sp extract has therapeutic efficacy against established malaria parasite. Acute toxicity of extract of *Xetospongia* sp observations that no death with up to an oral dose of 5000 mg kg^{-1} could indicate that the test extracts are very safe.

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