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Ethyl acetate Fraction of *Garcinia Mangostana* L Rind Study as Antimalaria and Antioxidant in *Plasmodium berghei* Inoculated Mice

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Abstract

BACKGROUND: Drug resistance to malaria is still a problem in various regions, and there have even been developments in resistance to the ACTs (artemisinin-based combination therapies) as standard antimalarial drugs included to artemisinin's partner drugs. Ethyl acetate fraction of *G. mangostana* L rind, containing xanthones as an antioxidant, has antimalarial activity in vitro which has a synergistic effect with artemisinin. That's why the activities of this fraction are needed to be studied in vivo.

AIM: To explore the antimalarial and antioxidant activity of ethyl acetate fraction of *Garcinia mangostana* L rind in mice.

METHODS: This was a complete randomised design true experimental study. Six groups of mice: a healthy mice group and 5 groups of *Plasmodium berghei* inoculated mice treated with various doses of the sample for 3 days compared to artemisinin. Parasitemia and total antioxidant status were examined and analysed using ANOVA, and probit analysis were done.

RESULTS: The parasitemia level in all of the treatment groups were lower than the positive control group without treatment ($p < 0.01$) and the parasitemia level was the lowest in artemisinin group which was not significantly different from the 100 mg/kg body weight dose group ($p > 0.05$). The parasitemia level in 20 and 4 mg/kg body weight dose groups were higher than the artemisinin group ($p < 0.01$). Parasite growth inhibition rate from the highest to the lowest consecutively was: artemisinin, 100 mg/kg body weight, 20 mg/kg body weight, 4 mg/kg body weight, and positive control group ($p < 0.05$) and ED50 was 3.396 mg/kg body weight. Total antioxidant status was the highest in 20 mg/ kg body weight dose and higher than the negative control group ($p < 0.05$) while the lowest total antioxidant status was in the positive control group.

CONCLUSION: Ethylacetate fraction of *G. mangostana* L rind potentially showed antimalarial and antioxidant activity in vivo. Further study is needed to explore the detail of its mechanism of action and its quantitative phytochemical analysis to find the leading compound in it.

Introduction

The main issue of malaria is the number of cases that are resistant to conventional antimalarial drugs. To overcome this issue, artemisinin-based combination regimen has been used. But resistance to the available artemisinin-based combination drugs has been reported in some countries [1]. Resistance also happens against artemisinin partner drugs such as piperaquine in dihydroartemisinin-piperaquine regimen in Cambodia [2], also against lumefantrine in artemether-lumefantrine regimen [3]. Therefore, it is necessary to find other alternative reserved drugs that could potentially be paired with artemisinin. Part of the mangosteen (*G. mangostana* L) fruit, i.e. the rind, contains xanthones [4]. A synthetic xanthone, i.e. 2, 3, 4, 5, 6-pentahydroxy xanthone prevent hemozoin formation by inhibiting heme polymerization [5]. Xanthones are also antioxidant and are possibly suitable for use in malaria because malaria decreases levels of antioxidant [6]. *In vitro*, it has been proven that some xanthone compounds worked synergistically with artemisinin as antimalaria against a 3D7 clone of *Plasmodium falciparum* [7]. The previous study showed that ethanolic extract and ethylacetate fraction of *G. mangostana* L rind had antimalarial activity and worked synergistically with artemisinin against a 3D7 clone of *P. falciparum in vitro* [8]. In the previous study, *in vivo* antimalarial effect of ethanolic extract of the *G. mangostana* L rind also has been studied against *P. berghei* inoculated mice [9] but there is no report about the antimalarial

activity of the ethyl acetate fraction *in vivo*. By the way, reported by other researchers that alphamangostin, which is an antioxidant, containing in this preparation caused this fraction to have SPF [10]. This fact supports the background of this study to explore the antioxidant activity besides the antimalarial activity of the *G. mangostana* L rind ethylacetate fraction *in vivo* in *P. berghei* inoculated mice.

Material and Methods

Ethical Issue

This study was approved by the ethics committee of Maranatha Christian University-Hospital Immanuel according to SK NO: 040/KEP/IV/2014 on the principle of 3 R (reduction, replacement, refinement).

Plant Sample

Mangosteen fruit was collected from Subang, West Java, Indonesia in March 2012, and has been identified by Djuandi, a curator at the Herbarium Bandungense, Sekolah Tinggi Imu Hayati, Bandung Institute of Technology (ITB), Bandung, Indonesia. The fruit was ripe with dark purple colour. The voucher specimen has been deposited by Dr J S Rahajoe in a publicly available herbarium, the Herbarium Bogoriense, Research Center of Biology, Indonesian Institute of Sciences in 2012 with deposition number of 1143/IPH.1.02/lf.8/VII/2012. After fruit washing, the rind has been proximately analysed previously [8].

G. mangostana L Rind extraction, fractionation, and treatment preparation

The rind was cut into small pieces, air dried, pulverised and macerated with 96% alcohol, followed by evaporation to obtain paste like extract according to the standard procedure [11]. The extract was macerated again with hexane and ethyl acetate consecutively using the same method to obtain paste like ethyl acetate fraction from the hexane fraction. This fraction was stored in -20°C freezer until used. For this study, the artemisinin, as well as this fraction, were dissolved in dimethyl sulfoxide (DMSO, Sigma Aldrich, IL, USA) to make a stock solution. Ethyl acetate fraction stock solution was diluted to adjust each of the treatment doses in 0.1 mL solution. The doses were 100 mg/kg body weight, 20 mg/kg body weight, and 4 mg/kg body weight daily. Artemisinin dose as a positive control dose was 50 mg/kg body weight/day in 0.1 mL.

Animals and treatment

A group of 24 DDY strain male mice, 8 weeks of age with 20-25 grams weight, were obtained from the Biopharma Institute, Bandung. They were divided into 6 treatment groups randomly in complete randomised design consisting of 5 groups of *P. berghei* inoculated, and 1 group of healthy non inoculated mice as a negative control group (NC). Each group contained 4 replications according to a certain formula from Hanafiah [12]. The mice were fed with pellets and water *ad libitum*. The *P. berghei* was obtained from the Malaria Laboratory, The Eijkman Insitute for Molecular Biology, Jakarta and was inoculated intraperitoneally into a donor mouse until the minimal parasitemia of 5-10%, and then this mouse was terminated by cervical dislocation, and cardiac puncture was done to obtain the parasites. All of the 24 experimental mice were adapted to the condition of biology laboratory for 1 week in condition: 12 hours light/ dark cycle, 23-24°C, 60-70% relative humidity, then each of the 20 mice of the 5 inoculated groups were inoculated intraperitoneally with 10^7 parasitized red blood cells (pRBC) from the donor in 200 µL PBS solution. After reaching about 5% parasitaemia, around the 4-5th day, these mice were divided into five groups.

Each of these groups was orally treated every day, once daily, for 3 days consecutively as follows: aquadest (the positive control group = PC), 50 mg/kg body weight/day of artemisinin (artemisinin control group = AC), 100 mg/kg body weight/day of ethyl acetate fraction (the first dose group = A1), 20 mg/kg body weight/ day of ethyl acetate fraction (second dose group = A2), and 4 mg/kg body weight/day of ethyl acetate fraction (third dose group = A3). Parasitaemia was calculated before and after the treatment on the fourth day by determining the parasites amount microscopically per 5,000 red blood cells in Giemsa stain thin blood smears. The rest 4 healthy non inoculated mice were used as a negative control (NC) for the antioxidant test. After completion of the experiment, all of these mice in six groups were terminated by neck dislocation, and their blood was taken from cardiac puncture for total antioxidant levels examination (total antioxidant status = TAS).

Total antioxidant status analysis

Each of the mice serum samples was analysed to determine total antioxidant status using Cayman Antioxidant Assay Kit accordingly. The absorbances were read at 750 nM by ELISA plate reader.

Statistical analysis

The data of parasitaemia and total antioxidant status were analysed using ANOVA followed by Tukey HSD ($\alpha = 0.05$) and ED_{50} (effective dose 50) of

the fraction as antimalaria was analyzed using probit analysis.

Results

The effect of the ethyl acetate fraction of G. mangostana L Rind against parasitaemia in mice suffering berghei malaria

The parasitemia level in the 5 treatment groups on the day before treatment, day 4 (after 3 days treatment), and parasite growth inhibition is shown in following Table 1.

Table 1: **Parasitemia level in percentage in the 5 treatment groups on the day before treatment, day 4 (after 3 days of treatment), and parasite growth inhibition**

Treatment Groups	Parasitemia level average ± stdev (%) before treatment	Parasitemia level average ± stdev (%) on day 4 (after 3 days of treatment)	Parasite growth inhibition average ±stdev (%)
PC	(5.22 ± 0.5)^{a}	(15.9 ± 3.34)^{a}	
AC	(5.32 ± 1.8)^{a}	(0.24 ± 0.11)^{b}	98 ± 0.01^{a}
A1	(5.42 ± 1.2)^{a}	(3.02 ± 0.33)^{b}	81 ± 0.02^{b}
A2	(5.3 ± 0.93)^{a}	(6.1 ± 0.75)^{c}	62 ± 0.05^{c}
A3	(5.2 ± 0.98)^{a}	(7.3 ± 0.85)^{c}	54 ± 0.05^{d}

Notes: the same alphabet superscript in each column shows no significant difference (p > 0.05); PC = mice suffering berghei malaria without treatment; AC = mice suffering berghei malaria treated with artemisinin 50 mg/kg body weight; A1 = mice suffering berghei malaria treated with 100 mg/kg body weight of ethylacetate fraction; A2 = mice suffering berghei malaria treated with 20 mg/kg body weight of ethyl acetate fraction; A3 = mice suffering berghei malaria treated with 4 mg/kg body weight of ethylacetate fraction.

Parasitemia levels of the five groups after 3 days of treatment (day 4) could be compared because each of the parasitemia levels on day 0 was no significant difference (p > 0.05). This was shown in the data of Figure 1.

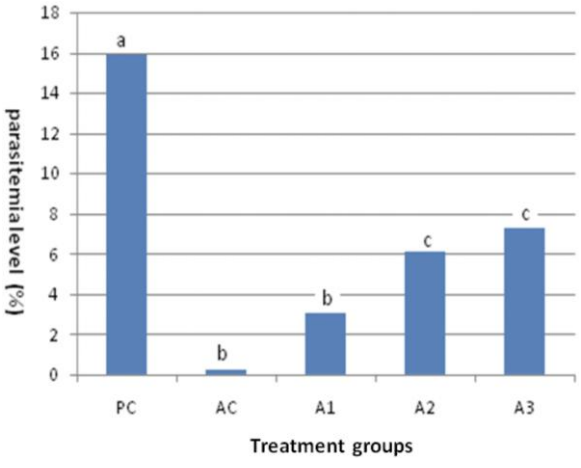

Figure 1: Parasitaemia level in percentage in the 5 treatment groups after 3 days of treatment (day 4). The same letter above each of the columns indicates that there is no significant difference (P > 0.05); PC = mice suffering berghei malaria without treatment; AC = mice suffering berghei malaria treated with artemisinin 50 mg/kg body weight; A1 = mice suffering berghei malaria treated with 100 mg/kg body weight of ethyl acetate fraction; A2 = mice suffering berghei malaria treated with 20 mg/kg body weight of ethyl acetate fraction; A3 = mice suffering berghei malaria treated with 4 mg/kg body weight of ethyl acetate fraction

Parasite growth inhibition rate after 3 days of treatment (day 4) indicates that the highest to the lowest group was: AC, A1, A2, and A3 consecutively (p < 0.05). This was shown in the following Figure 2.

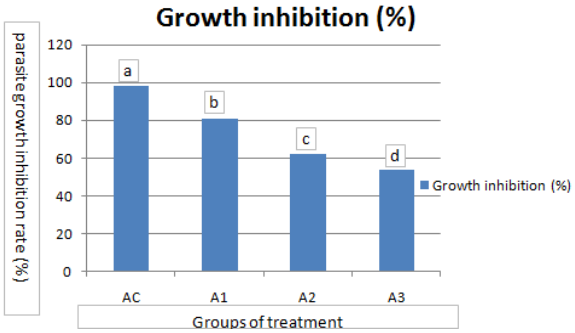

Figure 2: The parasite growth inhibition rate (%) in various treatment. The same letter above each of the columns indicates that there is no significant difference (P > 0.05); PC = mice suffering berghei malaria without treatment; AC = mice suffering berghei malaria treated with artemisinin 50 mg/kg body weight, A1 = mice suffering berghei malaria treated with 100 mg/kg body weight of ethyl acetate fraction; A2 = mice suffering berghei malaria treated with 20 mg/ kg body weight of ethylacetate fraction; A3 = mice suffering berghei malaria treated with 4 mg/kg body weight of ethyl acetate fraction

As shown in Figure 1, dose 1, dose 2, and dose 3 of ethyl acetate fraction of *G. mangostana* L rind showed antimalarial activity (p < 0.05) and dose 1 was the strongest one which had an equivalent antimalarial activity as artemisinin (p > 0.05).

ED_{50} (effective dose 50), which indicates the level of antimalarial activity could be calculated from the parasite growth inhibition data shown in Figure 2 using probit analysis. The correlation between the log of doses and probit of the percentage of parasite growth inhibition was shown in Figure 3.

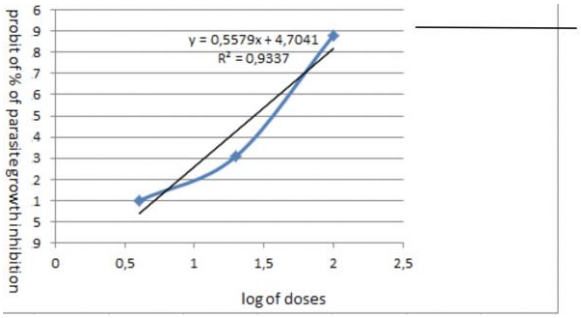

Figure 3: Correlation between the probit of the percentage of parasite growth inhibition and the log of doses. According to the formula shown in the figure, ED_{50} was 3.396 mg/kg body weight

Effect of the ethyl acetate fraction of G. mangostana L rind *against total antioxidant status in mice suffering berghei malaria*

Total antioxidant status in the sera of mice suffering berghei malaria after various treatments are shown in the following Table 2.

Table 2: Total antioxidant status in the sera of mice suffering berghei malaria after various treatments

	Total antioxidant level (mMTrolox)				Average±SD
PC	0.070	0.067	0.081	0.099	$0.079^{a} \pm 0.015$
NC	0.221	0.222	0.276	0.222	$0.235^{b} \pm 0.027$
AC	0.193	0.231	0.203	0.191	$0.204^{b} \pm 0.018$
A1	0.121	0.146	0.112	0.108	$0.122^{c} \pm 0.017$
A2	0.2316	0.2732	0.2393	0.3578	$0.276^{a} \pm 0.058$
A3	0.2046	0.2240	0.2039	0.2288	$0.215^{b} \pm 0.013$

Notes: the different superscript alphabet means that there is a significant difference ($p < 0.05$). NC = healthy mice without treatment; PC = mice suffering berghei malaria without treatment; AC = mice suffering berghei malaria treated with artemisinin 50 mg/kg body weight, A1 = mice suffering berghei malaria treated with 100 mg/kg body weight of ethyl acetate fraction; A2 = mice suffering berghei malaria treated with 20 mg/kg body weight of ethyl acetate fraction; A3 = mice suffering berghei malaria treated with 4 mg/kg body weight of ethyl acetate fraction; SD = standard deviation. The TAS level from the highest to the lowest consecutively was: A2, NC/AC/A3, A1, PC.

As shown in Table 2, the TAS level of ethyl acetate fraction groups was higher than PC group ($p < 0.05$) which meant that the fraction had antioxidant activity and could rise the TAS level and possibly could encounter the lack of antioxidant status in mice suffering malaria. The highest serum antioxidant level was in A2 treatment, not in A1. That's mean that there was an optimum dose of this fraction, which leads to the maximum level of the serum antioxidant status. This was consistent with our previous research on co-cultivation of 3D7 clone of *Plasmodium falciparum*-HUVEC (human umbilical vein endothelial cell) which were incubated with IC_{50} of artemisinin and various concentrations of L ascorbic acid as mentioned in the discussion chapter.

Discussion

Qualitative phytochemical analysis of ethyl acetate fraction of *G. mangostana* L rind was done according to the reported method. It contains several phytochemicals: tannins, terpenoids, triterpenoids, and flavonoids [13], [14].

The antimalarial activity of this ethyl acetate fraction of *G. mangostana* L rind *in vivo* supports the previous study indicating this antimalarial activity *in vitro* [8]. Similar *in vivo* study in Swiss Webster mice suffering berghei malaria showed that ethyl acetate fraction of *G. mangostana* L rind also had a very good antimalarial activity even more active than hexane or methanol fraction [15]. This antimalarial action of the ethyl acetate fraction of *G. mangostana* L rind may be caused by alphamangostin containing in it [10], and alphamangostin itself is a kind of xanthones which had good antimalarial action *in vitro* against 3D7 clone of *P. falciparum* working synergistically with artemisinin [7]. Xanthones could inhibit aggregation of heme, block hemozoin formation, and they bind to the soluble heme cause intra food vacuole osmotic pressure enhancement, and the lysis [5]. Transmission electron microscopical image also showed the inhibition of hemozoin formation of alphamangostin [16]. The result of this study is closely correlated with the qualitative phytochemical analysis, which is needed to be followed by quantitative analysis in further study. This antimalarial action of ethyl acetate fraction of *G. mangostana* L rind was supported the fact that besides gammamangostin, alphamangostin was the most abundant kind of xanthones in mangosteen rind [17].

According to *P. falciparum* foot printing study, mangosteen rind extract targeted several metabolic pathways, particularly glucose and TCA metabolisms [18].

According to Figure 3 and the ED_{50} of this ethyl acetate fraction against *P. berghei in vivo*, the fraction has potentially a very good antimalarial activity [19]. Further study is needed to observe this activity for a longer period for detecting the possibility of recrudescence. Treatment with higher dose is also needed to find out the dose that can inhibit 100% parasite growth.

The previous research indicated that L ascorbic acid as an antioxidant could improve the viability of HUVEC and produce the highest level of GSH at optimum supplementation and not at the maximum [20]. Another study also showed the similar result about the antioxidant as well as prooxidant effect of curcumin which was concentration dependent that's mean in low concentration it had antioxidant effect in contrast against high concentration which had pro-oxidant effect [21]. This phenomenon is very interesting to be studied further.

In conclusion, ethylacetate fraction of *G. mangostana* L rind potentially showed antimalarial and antioxidant activity *in vivo*. Further study is needed to explore the detail of its mechanism of action and its quantitative phytochemical analysis to find the leading compound in it. Longer period of parasitaemia observation and higher doses are also needed to detect recrudescence possibility and to explore the dose which can cause 100% inhibition of parasite growth.

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