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Antimalarial activity and toxicity of *Garcinia mangostana* Linn.

Ratchanu Bunyong, Wanna Chaijaroenkul, Tullayakorn Plengsuriyakarn, Kesara Na-Bangchang\*

Chulabhorn International College of Medicine, Thammasat University, Pathumthani, Thailand

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## ABSTRACT

**Objective:** To investigate the antimalarial activity and toxicity of the crude ethanolic extract of its pericarp both *in vitro* and *in vivo*. **Methods:** The antimalarial activity of *Garcinia mangostana* (*G. mangostana*) Linn. extract against 3D7 and K1 *Plasmodium falciparum* (*P. falciparum*) clone were assessed using SYBR green I –based assay. A 4–day suppressive test of *Plasmodium berghei* (*P. berghei*) infected mouse was performed to investigate *in vivo* antimalarial activity. **Results:** The *in vitro* antimalarial activity was selective (SI > 5) and classified as weak and good to moderate activity against both 3D7 and K1 *P. falciparum* clones with median IC<sub>50</sub> (range) values of 11.12 (10.94–11.29) and 7.54 (6.80–7.68)  $\mu$ g/mL, respectively. The extract was considered non-toxic to mice. The maximum tolerated doses for acute and subacute toxicity in mice were 5 000 and 2 000 mg/kg, respectively. Median (range) parasite density on day 4 of the negative control group (25% Tween–80), mice treated with 250, 500, 1000, and 2 000 mg/kg body weight of the extract, and 10 mg/kg body weight of chloroquine for 14 d were 12.8 (12.2–13.7), 11.4 (9.49–13.8), 11.6 (9.9–12.5), 11.7 (10.6–12.8), 10.9 (9.4–11.6) and 0 (0–0)% respectively. Parasite density on day 4 in the control group treated with Tween–80 was higher than the groups treated with chloroquine and all dose levels of the extract. **Conclusions:** *G. mangostana* Linn. showed weak antimalarial activity of the extract both *in vitro* and *in vivo* could be due to limitation of absorption of the active compounds.

## 1. Introduction

Malaria remains one of the most important infectious diseases in the world. It constitutes a public health problem in more than 90 countries inhabited by about 40% of the world's population. Statistics of the World Health Organization in 2012 showed an estimate of about 274 million malaria cases annually with approximately 1.1 million deaths, mostly in children under five years old[1]. Of the five species of human malarial parasites, *Plasmodium falciparum* (*P. falciparum*) is remarkable for its high case fatality rate and resistance developed to existing antimalarial drugs. The emergence and spread of multidrug resistant *P. falciparum* continue to be a major public health problem in the management and control of malaria in

several endemic countries. This situation was particularly threatening until the introduction of artemisinin–based combination therapies (ACTs)[2]. It is being debated whether *P. falciparum* has developed resistance to artesunate–mefloquine combination, one of the most commonly used ACTs. The increase in fifty percent inhibitory concentration (IC<sub>50</sub>) and a delay in parasite clearance have been observed following treatment with this combination[3–5]. The observed delayed parasite clearance suggests that some parasites can survive the treatment for a longer period of time than expected, although they are eventually killed by the drugs. This raises the possibility of a unique survival mechanism different from a classical drug–resistance phenotype. New effective and affordable antimalarial drugs are therefore urgently needed. Plants are important sources of medicines including antimalarial drugs as in the cases of quinine and artemisinins[6]. The aim of the study was to assess the antimalarial activity (*in vitro* and *in vivo*) and toxicity of the crude ethanolic extract of *Garcinia mangostana* (*G. mangostana*) Linn. or mangosteen. This fruit pericarp has been used in traditional medicine in several Asian countries for several purposes including treatment of skin infections and wounds[7,8].

\*Corresponding author: Prof. Dr. Kesara Na-Bangchang, Chulabhorn International College of Medicine, Thammasat University (Rangsit Campus), Pathumthani 12121, Thailand.

Tel: +662-564-4440-79 Ext.1803

Fax: +662-564-4398

E-mail: kesaratmu@yahoo.com

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## 2. Materials and methods

### 2.1. Plant materials and preparation of the crude ethanolic extract of *G. mangostana* Linn.

*G. mangostana* Linn. fruits were purchased from Talard–Tai Market in Pathumthani Province of Thailand. The fruit was rinsed thoroughly with tap water to remove extraneous contaminants and the pericarp was cut into small pieces, dried at 45 °C and ground into powder with pestle and mortar. Extraction was carried out by macerating the powder plant materials (100 g) in a flask containing 500 mL of 95% ethanol (at 25–30 °C) for 7 d. The extraction solvent was separated, filtered (through Whatman no.1 filter paper) and evaporated under reduced pressure by rotary evaporation. The ethanolic extract yield of 400 g dried weight of mangosteen pericarp powder was 10.55% (w/w).

### 2.2. Determination of fingerprint of the crude ethanolic extract of *G. mangostana* Linn.

Fingerprint of the crude ethanolic extract of *G. mangostana* Linn. pericarp was analyzed by HPLC using  $\alpha$  – mangostin as a chemical marker<sup>[9]</sup>. The HPLC system consists of a solvent delivery system (Spectra System P4000 Quaternary Solvent Delivery/Controller: Thermo Fisher Scientific, California, USA), equipped with solvent degasser (SpectraSystem SCM1000 Solvent Degasser: Thermo Fisher Scientific, California, USA), an auto sampler (SpectraSystem AS3500: Thermo Fisher Scientific, California, USA), and a UV detector (SpectraSystem UV/Vis3000: Thermo Fisher Scientific, California, USA). The optimal wave length was set at 254 nm. The separation was carried out on a reversed–phase column (Thermo Hypersil Gold C18, 250 mm×2.1 mm i.d., 5  $\mu$  m: Thermo Scientific, California, USA). The elution solvent consisted of water (A) and acetonitrile (B) running at a flow rate of 1.0 mL/min. Total running time was 37 min and the gradient mode was as follow: 55% B to 85% B for 0–30 min, constant at 85% B for 5 min, 85% B to 55% B for 1 min, and constant at 55% B for 4 min. The chromatographic analysis was operated at 25 °C. An aliquot of 20  $\mu$  L of sample or standard solution was injected into the column.

### 2.3. In vitro experiment

#### 2.3.1. Parasite culture

*P. falciparum* chloroquine–resistant K1 and chloroquine–sensitive 3D7 clones were kindly provided by the Malaria Research Unit, Institute of Health Research, Chulalongkorn University, Thailand. Both clones were maintained in continuous culture in O+ human erythrocytes suspended in RPMI 1640 (Gibco, California, USA) culture medium supplemented with 10% human B serum and 25 mM HEPES (at 37 °C under a gas mixture of 5% CO<sub>2</sub>, 5% O<sub>2</sub> and 90% N<sub>2</sub>) according to the standard method described by Trager and Jensen<sup>[10]</sup>. The level of parasitemia in the culture was

maintained between 2 and 10%. Synchronization of the parasite culture to ring stage *P. falciparum* was performed using 5% sorbitol<sup>[11]</sup>.

#### 2.3.2. Assessment of antimalarial activity in vitro

Antimalarial activity of the crude ethanolic extract of *G. mangostana* Linn. pericarp was investigated *in vitro* using SYBR Green I assay<sup>[12,13]</sup>. Highly synchronous ring stage parasite was used in each assay. An aliquot of parasite inoculum (50  $\mu$  L) with 2% parasitemia and 1% hematocrit was added into each well of microtiter plate. The extract (dissolved in dimethyl sulfoxide or DMSO and diluted with RPMI 1640 to final concentration of 1%) was added to the malaria culture at eight final concentrations of 0.78, 1.56, 3.13, 6.25, 12.5, 25, 50 and 100  $\mu$ g/mL. Chloroquine (3.89–498.15 nM) and artesunate (0.39–50.0 nM) were used as standard antimalarial drugs. The experiment was repeated three times (triplicate each). IC<sub>50</sub> value (drug concentration that inhibits the parasite growth by 50%) was used as an indicator of antimalarial activity and was determined from a log–dose response curve plotted using the Calcsyn™ version 1.1 (BioSoft, Cambridge, UK).

#### 2.3.3. In vitro assay for cytotoxic activity

Normal human epithelial cell (HRE) was purchased from Promocell Co. Ltd. (Heidelberg, Germany) and cultured in renal epithelial cell growth medium 2 supplemented with SupplementPack (Promocell Co. Ltd., Heidelberg, Germany). The cell was incubated at 37 °C in 5% CO<sub>2</sub> atmosphere with 95% humidity and was seeded in a 96–well plate at a density of 10<sup>4</sup> cells/well in 100  $\mu$  L culture medium. Following 24 h incubation and attachment, cells were treated with varying concentrations of each plant extract. The concentration range of the extract used was 1.95–250.00  $\mu$ g/mL. After 48 h incubation, following washing and incubation with MTT [3–(4,5–dimethylthiazol–2–yl)–2,5–diphenyltetrazolium bromidesolution, 20  $\mu$  L of 5 mg/mL] at 37 °C for 3 h, cells were lysed with DMSO. The yellow MTT dye was reduced by succinic dehydrogenase in the mitochondria of viable cells to purple formazan crystals. Absorbance was measured at 570 nm using a microplate reader (Variokan, Thermo, USA). Experiments were repeated three times, triplicate for each experiment). The IC<sub>50</sub> values were determined using Calcsyn™ software (BioSoft, Cambridge, UK). The selectivity index (SI) was calculated from the IC<sub>50</sub> ratio of each extract obtained from HRE cell and K1 or 3D7 clone.

### 2.4. In vivo experiments

#### 2.4.1. Animals

Imprinting control region (ICR) mice (5–7 weeks of age, weighting 20–40 g) of both genders were used in the study. All were obtained from the National Laboratory Animal Centre, Thailand. Animal experiments were carried out in accordance with the OECD Guideline for Chemicals. The

animals were housed under standard conditions and fed with a stock diet and water ad libitum. Approval of the study protocol was obtained from the Ethics Committee for Animal Research, Thammasat University, Thailand.

#### 2.4.2. Toxicity tests

The ethanolic extract of *G. mangostana* Linn. (pericarp) was weighted and resuspended with 25% Tween–80 to obtain the desired concentrations. ICR mice were fasting 2 h before feeding with the extract. Animals were divided into four groups of six (3 males and 3 females for each group). For the acute toxicity test, mice in each group were fed with the extract at a single oral dose of 5 000 mg/kg body weight; control group received a single oral dose of 25% Tween–80 (1 mL). For the subacute toxicity test, mice in each group were fed with the extract at a daily oral dose of 2 000 mg/kg body weight for 14 d; control group received a daily oral dose 1 mL of 25% Tween–80 for 14 d. General behavior of each mouse was observed continuously for 1 h after each dose, intermittently every 4 h, and thereafter over a period of 24 h<sup>[14]</sup>. Animals were observed for up to 14 d for the acute toxicity test and 28 d for the subacute toxicity test for any sign of toxicity (behavioral change related to central nervous, cardiovascular and gastrointestinal systems as well as complete blood count, liver and kidney function tests), body weight change and water and food consumption. At the end of the observational period, all animals were sacrificed under ether anesthesia and vital organs (heart, lung, liver, spleen and kidney) were removed from all animals for gross and histopathological examination.

#### 2.4.3. Assessment of antimalarial activity of plumbagin in *Plasmodium berghei* (*P. berghei*)–infected mouse model (4–day suppressive test)

The *in vivo* antimalarial activity of the ethanolic extract of *G. mangostana* Linn. was evaluated using a 4–day suppressive test in *P. berghei*–infected mouse model<sup>[15]</sup>. *P. berghei* (ANKA) strain used in the experiment was obtained from the National Center for Genetic Engineering and Biotechnology (BIOTEC), Thailand. The parasite had been maintained by serial blood passage in mice, and blood stage stored at –196 °C until use.

ICR mice were divided into six groups (3 males and 3 females for each group). The donor mice were infected with 200 µL of *P. berghei* parasite inoculum. The parasitized blood of each donor mouse was collected from the tail vein and diluted with 0.9% sodium chloride. Mice were infected with saline suspension of  $1 \times 10^7$  parasitized erythrocytes (0.2 mL) by intraperitoneal injection (Day 0). Four hours after infection, animals were treated with the ethanolic extract of *G. mangostana* Linn. at oral daily doses of 250, 500, 1 000 or 2 000 mg/kg body weight for four consecutive days (test group 1, 2, 3 and 4, respectively). Positive and negative control groups were fed with the antimalarial chloroquine at oral daily doses of 10 mg/kg body weight and 25% Tween–80, respectively. On day 4 (96 h post infection), parasitemia of

individual mouse was determined under light microscope by examination of giemsa–stained thin blood smears prepared from mouse tail blood<sup>[16]</sup>. The mean parasitemia in each group of mice was used to calculate the % suppression for each dose using the formula:

$$\frac{\text{Parasitemia of negative control} - \text{Parasitemia of test}}{\text{Parasitemia of negative control}}$$

The antimalarial activity of the ethanolic extract of *G. mangostana* Linn. was determined from the ratio of percentage of parasite reduction in treated and negative control groups<sup>[17]</sup>. Results are expressed as median (range) values. Comparison of difference in quantitative variables between more than two and two groups was performed using Kruskal Wallis and Mann–Whitney *U* tests (SPSS version 12.0, SPSS Inc., California, USA). Statistical significance level was set at  $\alpha < 0.05$  for all tests.

### 3. Results

#### 3.1. *In vitro* antimalarial activities of *G. mangostana* Linn.

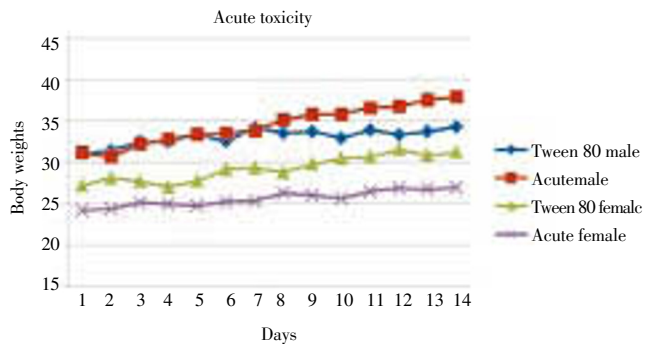
The antimalarial activity of the ethanolic extract of *G. mangostana* Linn. against 3D7 chloroquine–sensitive and K1 chloroquine–resistant *P. falciparum* clones were categorized as weak and good to moderate with median IC<sub>50</sub> (range) values of 11.12 (10.94–11.29) and 7.54 (6.80–7.68) g/mL, respectively. The corresponding IC<sub>50</sub> values for chloroquine and artesunate were 10.5 *vs.* 128.7 and 2.1 *vs.* 1.91 nM, respectively.

Cytotoxicity assay was performed using normal human HRE cell line in order to evaluate the selectivity of antimalarial activity of the crude ethanolic extract of *G. mangostana* Linn. The median (range) IC<sub>50</sub> value was 67.3 (58.00–71.60) µg/mL. The antimalarial activity of the extract against both clones were found to be selective, with SI values of 6.04 and 8.90, respectively.

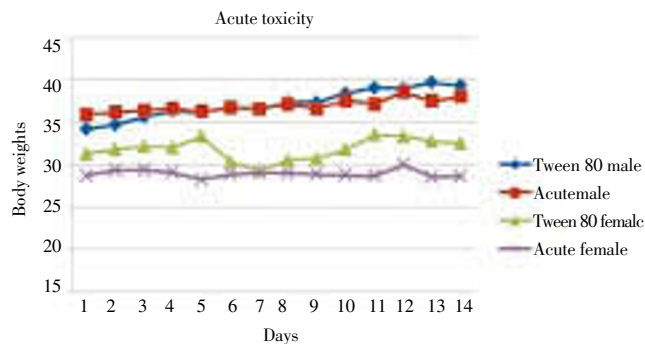
#### 3.2. Toxicity tests

The toxicity of the ethanolic extract of *G. mangostana* Linn. when given as a single oral dose (acute toxicity) and 14–day daily doses (subacute toxicity) in mice was investigated in order to define optimal dose to be used for evaluation of its *in vivo* antimalarial activity in malarial mouse model. Results indicated virtually no toxicity of *G. mangostana* Linn. at a maximum single oral dose of 5 000 mg/kg body weight (acute toxicity, Figure 1) and daily oral doses of 2 000 mg/kg body weight for 14 d (subacute toxicity, Figure 2). All mice survived and there was neither sign of toxicity nor significant change in water and food consumption and body weights of mice in both groups during the 14 d observation period. The gross examination of vital organs,

i.e., heart, lung, liver, spleen and kidney in both treated and control groups were similar either in size and cell morphology.



**Figure 1.** Median body weights of male and female mice ( $n=3$  each) during the first 14 d following the administration of a single oral dose of 5 000 mg/kg body weight of the crude ethanolic extract of *G. mangostana* Linn. pericarp compared with 25% Tween 80 (negative control) in acute toxicity test.



**Figure 2.** Median body weights of male and female mice ( $n=3$  each) during the first 14 d following the administration of daily oral doses of 2 000 mg/kg body weight of the crude ethanolic extract of *G. mangostana* Linn. pericarp for 28 d, compared with 25% Tween 80 (negative control) in subacute toxicity test.

### 3.3. Antimalarial activity of *G. mangostana* Linn. in *P. berghei*-infected mouse model (4-day suppressive test)

Results of the 4-day suppressive antimalarial test of the

ethanolic extract of *G. mangostana* Linn. and chloroquine in mice infected with *P. berghei* ANKA strain are summarized in Table 1. Median (range) parasite density on day 4 of the negative control group (25% Tween-80), mice treated with 250, 500, 1 000 and 2 000 mg/kg body weight of the extract, and 10 mg/kg body weight of chloroquine for 14 d were 12.8 (12.2–13.7), 11.4 (9.49–13.8), 11.6 (9.9–12.5), 11.7 (10.6–12.8), 10.9 (9.4–11.6) and 0 (0–0)% respectively. Parasite density on day 4 in the control group treated with Tween-80 was higher than the groups treated with chloroquine and all dose levels of the extract. Chloroquine exhibited the most potent antimalarial activity with respect to its activity on reduction of parasitemia on day 4 and prolongation of survival time. Parasite density (%) on day 4 following chloroquine treatment (0%) was significantly lower than 25% Tween 80 (negative control) and the extract at all dose levels. In addition, parasite suppression (%) on day 4 of mice treated with chloroquine (100%) was significantly higher than the negative control group (0%) and the groups treated with the extract at the doses of 2 000 (14.4%), 1 000 (8.5%), 500 (9.3%) and 250 (10.5%) mg/kg body weight. The survival time in the group treated with chloroquine was also significantly shorter than the negative control and the groups treated with the extract at all dose levels. Parasitemia of mice treated with chloroquine started to rise on day 5 (0.20%) until day 8 (24.11%) of treatment, while that was rising between 19%–29% in mice treated with the extract (Table 2).

## 4. Discussion

The *in vitro* antimalarial activity against K1 and 3D7 *P. falciparum* clones of the crude ethanolic extract of *G. mangostana* Linn. pericarp was categorized into good to moderate and weak to antimalarial activities with median  $IC_{50}$  values of 7.54, and 11.12  $\mu$ g/mL, respectively. These findings were in agreement with that reported by Thiengsusuk and colleagues, where the  $IC_{50}$  of the extract against K1 and 3D7 *P. falciparum* clones were reported as

**Table 1**

*In vivo* antimalarial activities of the ethanolic extract of *G. mangostana* Linn. pericarp against *P. berghei* ANKA strain.

Treatment	Dose (mg/kg)	Median (range: %) of parasite density on Day 4	% Inhibition/ Suppression on Day 4	Median (range: days) of survival time
25% Tween 80	–	12.8 (12.2–13.7) <sup>a</sup>	0.0	17 (15–18) <sup>d</sup>
<i>G. mangostana</i> Linn.	2 000	10.9 (9.4–11.6)	14.4	15 (9–17)
<i>G. mangostana</i> Linn.	1 000	11.7 (10.6–12.8)	8.5	16 (10–17)
<i>G. mangostana</i> Linn.	500	11.6 (9.9–12.5)	9.3	16 (9–20)
<i>G. mangostana</i> Linn.	250	11.4 (9.49–13.8)	10.5	15 (8–17)
Chloroquine	10	0.0 <sup>b</sup>	100.0 <sup>c</sup>	10 (10–12)

Data are presented as median (range),  $n = 6$ . <sup>a</sup>Significantly higher than mice treated with 2 000, 1 000, 500, and 250 mg/kg of the extract ( $P<0.05$ ); <sup>b</sup>Significantly lower than negative control (25% Tween 80), and mice treated with 2 000, 1 000, 500 and 250 mg/kg body weight of the extract ( $P=0.002$ ); <sup>c</sup>Significantly higher than mice treated with 2 000, 1 000, 500, and 250 mg/kg body weight of the extract ( $P=0.02$ );

<sup>d</sup>Significantly higher than mice treated with 2 000 ( $P=0.029$ ), 1 000 ( $P=0.037$ ), 500 ( $P=0.030$ ), and 250 ( $P=0.031$ ) mg/kg body weight of the extract, and mice treated with 10 mg/kg body weight chloroquine ( $P=0.002$ ).

**Table 2**

In vivo antimalarial activity (represented by % parasite density on Day 5, 6 and 8) of the crude ethanolic extract of *G. mangostana* Linn. pericarp against ANKA strain *P. berghei*.

Treatment	Dose (mg/kg)	Parasite density (%)		
		Day 5	Day 6	Day 8
25% Tween 80	–	21.44	18.40	20.62
<i>G. mangostana</i> Linn.	2 000	27.27	23.68	20.33
<i>G. mangostana</i> Linn.	1 000	28.76	26.21	21.50
<i>G. mangostana</i> Linn.	500	21.81	21.86	20.51
<i>G. mangostana</i> Linn.	250	23.04	28.28	19.25
Chloroquine	10	0.20	0.67	24.11

3.6 and 11.8  $\mu$ g/mL, respectively<sup>[18]</sup>. *G. mangostana* Linn. pericarp has been shown to contain phenolic compounds such as condensed tannins, anthocyanins and xanthone derivatives<sup>[19–21]</sup>. Over 68 xanthone-type compounds have been isolated including the major constituent  $\alpha$ -mangostin, as well as  $\beta$ -mangostin,  $\gamma$ -mangostin, gartanin, gartanin and 9 hydroxycalabaxanthone<sup>[22]</sup>. Among them,  $\alpha$ -mangostin appears to be the most potent compound against both chloroquine-resistant and chloroquine-sensitive *P. falciparum* clones with  $IC_{50}$  ranging from 3.7 to 20  $\mu$ M<sup>[23]</sup>. Both  $\alpha$ -mangostin and  $\beta$ -mangostin were shown to exert inhibitory effects on the growth of D6 *P. falciparum* clone (chloroquine-sensitive) with mean  $IC_{50}$  of 5.1 and 7.0  $\mu$ M, respectively<sup>[24]</sup>. A series of oxygenated xanthenes was synthesized and their antimalarial activities were evaluated using a 4-day suppressive assay against ANKA *P. berghei* strain in BALB/c mice at repeated doses of 20 mg/kg body weight/d. The most active compound was 1, 3, 6, 8-tetrahydroxanthone, which reduced the percentage of erythrocytes infected by 70.5%, followed by norlichexanthone (44.3%) and its isomer 1,3,8-trihydroxy-6-methylxanthone (37.0%). Di-C-allyl-dihydroxanthone showed notable activity but relatively weaker (33.4%) and 1,3-dihydroxanthone exhibited very weak activity (15.1%)<sup>[25]</sup>.

Result of the *in vivo* antimalarial activity based on the standard 4-day suppressive test shown in the present study was also in accordance with the *in vitro* test showing moderate antimalarial activity of the ethanolic extract at the minimum dose of 250 mg/kg body weight/day. The mechanism of inhibition of *P. falciparum* growth by mangostin and derivatives is still unclear. However, it has been suggested that hydroxanthones exert their antiplasmodium activity through formation of soluble complexes with heme and thereby inhibiting parasite hemozoin formation, a process by which the malaria parasite protects itself against toxic effects of heme released following digestion of hemoglobin<sup>[24,26]</sup>. The extract was well tolerated when given to mice either at a single or repeated doses of up to 5 000 and 2 000 mg/kg body weight, respectively. Jujan and colleagues also reported no toxic effect of this plant in rats following a single oral dose of 5 000 mg/kg body weight<sup>[27]</sup>. In addition, a 28-day oral toxicity test at the dose of 1 000 mg/kg body weight/day did not produce any significant

dose-related changes on hematological parameters, serum biochemistry or histopathology of liver, kidney, lung, heart, spleen, adrenal gland, thymus, stomach and duodenum, small intestine, ovary, uterus, testis, epididymis muscle and nerve, thoracic spine eye, and brain<sup>[27]</sup>. In other studies, the crude extract (200 mg/kg body weight) and  $\alpha$ -Mangostin were shown to increase serum glutamic oxaloacetic and serum glutamic pyruvic transaminase in mice and rats<sup>[28]</sup>.

It was noted however that the survival time of mice treated with the extract at all dose levels including chloroquine observed in the present study were significantly shorter than the negative control. For chloroquine, this observation could be due to an increase in malaria disease severity as a consequence of rapid rise of parasitemia after day 5. It is possible that the antimalarial activity of the crude ethanolic extract of *G. mangostana* Linn. could be compromised by other inactive components. Furthermore, permeability of the active ingredients—and  $\beta$ -mangostin across intestinal membrane (Caco-2 cell) was shown to be extremely low (unpublished observation) and the oral bioavailability of  $\alpha$ -mangostin was also reported to be markedly low in rats<sup>[29]</sup>. Evidence of extensive first-pass metabolism of  $\alpha$ -mangostin<sup>[30]</sup> was provided in rats with an initial low plasma concentration followed by a rapid drop following oral dose administration. This pharmacokinetic factor would have led to an inadequate plasma concentration of this active moiety to suppress the parasite. Chemical derivatization of –and/or  $\beta$ -mangostin, or preparation of modified formulation such as liposomal formulation is required to improve their systemic bioavailability.

### Conflict of interest statement

We declare that we have no conflict of interest.

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