

Research Article

Antimalarial Activity of Kaempferol and Its Combination with Chloroquine in *Plasmodium berghei* Infection in Mice

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The search for new antimalarial drugs has become an urgent requirement due to resistance to the available drugs and the lack of an effective vaccine. In this respect, the present study aimed to evaluate the antimalarial activity of kaempferol against *Plasmodium berghei* infection in mice as an *in vivo* model. Chronic toxicity and antimalarial activities of kaempferol alone and in combination with chloroquine were investigated in *P. berghei* ANKA infected ICR mice using standard procedures. The results showed that chronic administration of 2,000 mg/kg of kaempferol resulted in no overt signs of toxicity as well as no hepatotoxicity, nephrotoxicity, or hematotoxicity. Interestingly, kaempferol exerted significant ($P < 0.05$) chemosuppressive, chemoprophylactic, and curative activities in a dose-dependent manner. The highest antimalarial activity was found at a dose of 20 mg/kg which resulted in a significantly ($P < 0.05$) prolonged survival of infected mice. Moreover, combination treatment of chloroquine and kaempferol also presented significant ($P < 0.05$) antimalarial effects, although the effects were not significantly different from the chloroquine treated group. From the results of the present study, it can be concluded that kaempferol possesses acceptable antimalarial activities. However, further investigation should be undertaken on the mechanism responsible for the observed antimalarial activity.

1. Introduction

Malaria is still a public health problem and it is among the most deadly parasitic diseases around the world especially in many tropical and subtropical regions. Malaria is a disease that is transmitted by the bite of a female *Anopheles* mosquito, which is infected by a parasite of the genus *Plasmodium*. There are an estimated 212 million cases of malaria globally which leads to some 445,000 deaths, most of which occur in African children under the age of five [1]. Although an effective vaccine is the best long-term control for malaria, the research on vaccine development is still at a preclinical stage and it is predicted that a malarial vaccine is still several years away [2]. However, the emergence of *Plasmodium* parasite resistance to existing antimalarial drugs, as well as *Anopheles* mosquito resistance to insecticides could render some of the current management tools ineffective and trigger a new rise in malaria mortality [3]. Hence, it is necessary to search for new, safe, and affordable antimalarial drugs for the treatment

of the disease. In this respect, medicinal plants are potential resources in the search for antimalarial agents. This is because most of these plants are rich in secondary metabolites such as flavonoids, terpenoids, alkaloids, and quercetin that have been reported to have antimalarial activity [4].

Kaempferol is a natural flavonoid mostly found in tea, broccoli, apples, strawberries, and beans. It has been reported and hypothesized to be an active compound that has several activities including antioxidant, anti-inflammation, antimicrobial, antiparasite, and anticancer [5]. In addition, kaempferol has also been described to possess hepatoprotective and immunomodulatory properties [6, 7]. Previously, kaempferol isolated from the leaves of *Schima wallichii* was shown to exert a potent antimalarial activity against chloroquine-resistant *P. falciparum* [8]. However, any antimalarial activity of kaempferol against *P. berghei* and particularly in combination with chloroquine has not yet been reported. Therefore, the aim of the present study was to assess the antimalarial activity of kaempferol both alone and in

combination with chloroquine using *P. berghei* infected mice as an *in vivo* model.

2. Materials and Methods

2.1. Chemicals. Chloroquine diphosphate salt (CQ) and kaempferol used in this study were purchased from Sigma (Sigma, Chemical, St. Louis, MO, US). All reagents were analytical grade and procured from certificated suppliers.

2.2. Drug Preparation. CQ (10 mg/kg) and kaempferol (1-2,000 mg/kg) were freshly prepared corresponding to the body weight of the experimental mouse in 0.2 ml of distilled water (DW) and orally administered by intragastric gavage.

2.3. Experimental Mice. Female ICR mice, 4-6 weeks of age, weighting between 25 and 35 g, were used for all experiments. The experimental mice were obtained from the National Laboratory Animal Center, Mahidol University, Thailand. They were kept at $25 \pm 2^\circ\text{C}$ with a 12 h photoperiod per day and provided with pellet diet and clean water *ad libitum*. All protocols were carried out in accordance with the guidelines of international animal care and welfare. All the animal experiments were approved by the Animal Ethic Committee of Walailak University (WU006/2018).

2.4. Chronic Oral Toxicity Assay. The chronic oral toxicity of kaempferol was evaluated according to the procedure guidelines of the Organization for Economic Co-Operation and Development (OECD) [9]. Two groups of naïve ICR mice (3 mice per group) were used. One group was administered with 2,000 mg/kg of kaempferol, while the second group received distilled water (DW) as a control, daily for 30 consecutive days. The mice were observed for signs of toxicity which included (but were not limited to) salivation, paw licking, weakness, stretching of the entire body, respiratory distress, coma, and death in the first three hours, and subsequently daily for 30 days. For evaluation of the effect of kaempferol on mouse hematological and serum parameters, mouse blood was collected by cardiac puncture into heparinized vacuum tubes on day 31 after treatment. The complete blood count

(CBC) and serum parameters for blood chemistry including aspartate aminotransferase (AST), alanine aminotransferase (ALT), blood urea nitrogen (BUN), and creatinine were measured using automated analyzers (IDXX ProCyt Dx and Cobas c311, respectively).

2.5. *Plasmodium berghei*. Chloroquine-sensitive *Plasmodium berghei* ANKA strain (PbANKA) was used for the induction of malaria in the experimental ICR mice. This parasite can be obtained from the Malaria Research and Reference Reagent Resource Center (MR4; <https://www.beiresources.org/Home.aspx>). Mice infected with PbANKA were used as the donor source. The parasites were subsequently maintained by serial passage of blood from the donor infected mice to naïve mice via intraperitoneal (IP) injection on a weekly basis. The donor infected mice with a parasitemia of 20-30% were sacrificed, and blood was collected by cardiac puncture into heparinized tubes. The blood was subsequently diluted with 0.9% normal saline solution (NSS) and infection undertaken by injecting 0.2 ml of the diluted blood which contained 1×10^7 parasitized erythrocytes via IP injection. Parasitemia was monitored daily by microscopic examination of Giemsa stained thin blood smears, and parasitemia was calculated using the following formula.

$$\begin{aligned} \text{\% parasitemia} \\ = \frac{\text{Number of parasitized erythrocytes} \times 100}{\text{Total number of erythrocytes}} \end{aligned} \quad (1)$$

2.6. Evaluation of Suppressive Antimalarial Activity of Kaempferol. Evaluation of the chemosuppressive antimalarial activity of kaempferol was carried out using the standard 4-day suppressive test as previously described [10]. Groups of ICR mice (3 mice per group) were inoculated with 1×10^7 PbANKA parasitized erythrocytes by IP injection. Two hours later, mice were administered 1, 10, and 20 mg/kg kaempferol by intragastric gavage followed by further administration once a day for 4 consecutive days (day 0-3). The untreated control mice were given 10 ml/kg of DW while the positive control mice were treated with 10 mg/kg of CQ. Moreover, the combination of 10 mg/kg of CQ and 20 mg/kg of kaempferol was also investigated. At day 4, parasitemia was determined and percent inhibition was then calculated using the following formula.

$$\text{\% inhibition} = \left(\frac{\text{\% parasitemia of untreated control} - \text{\% parasitemia of treated group}}{\text{\% parasitemia of untreated control}} \right) \times 100 \quad (2)$$

2.7. Evaluation of Prophylactic Antimalarial Activity of Kaempferol. Evaluation of the chemoprophylactic potential of kaempferol was performed according to the method previously described [11]. Groups of ICR mice (3 mice per group) were administered 1, 10, and 20 mg/kg kaempferol by intragastric gavage once a day for 4 consecutive days (day 0-3). Untreated control mice were given 10 ml/kg of DW while

the positive control mice were treated with 10 mg/kg CQ. Moreover, the combination of 10 mg/kg of CQ and 20 mg/kg of kaempferol was also administered. At day 4, the mice were inoculated with 1×10^7 PbANKA parasitized erythrocytes by IP injection. Seventy-two hours later, parasitemia was determined and percent inhibition was then calculated as described above.

TABLE 1: Chronic oral toxicity of kaempferol in mice.

Parameter	Control (10 ml/kg of DW)	2,000 mg/kg of KMF
Liver function test		
AST (U/L)	39.07 ± 0.42	35.23 ± 0.72
ALT (U/L)	13.75 ± 0.32	13.25 ± 0.22
Renal function test		
BUN (mg/dl)	45.55 ± 7.12	40.42 ± 2.34
Creatinine (mg/dl)	1.74 ± 0.31	1.57 ± 0.82
Complete blood cells		
Erythrocyte count (cells/mm ³)	6.13x10 ⁶ ± 5.43 x 10 ⁵	6.23x10 ⁶ ± 5.23x10 ⁵
Leucocyte count (cell/mm ³)	1,618 ± 43.21	1,621 ± 25.67
Neutrophils (%)	35.45 ± 0.61	37.74 ± 0.85
Eosinophils (%)	2.14 ± 0.58	3.25 ± 0.45
Basophils (%)	4.10 ± 0.36	4.01 ± 0.55
Monocytes (%)	6.99 ± 0.35	6.56 ± 0.24
Lymphocytes (%)	56.00 ± 1.47	56.67 ± 2.47

2.8. Evaluation of Curative Antimalarial Activity of Kaempferol. Evaluation of curative activity of kaempferol was carried out using the method previously described [12]. Groups of ICR mice (3 mice per group) were inoculated with 1x10⁷ PbANKA parasitized erythrocytes by IP injection. Seventy-two hours later, these mice were treated with 1, 10, and 20 mg/kg kaempferol administered by intragastric gavage once a day for 4 consecutive days (days 0-3). Untreated control mice were given 10 ml/kg of DW while the positive control mice were treated with 10 mg/kg of CQ. Moreover, the combination of 10 mg/kg of CQ and 20 mg/kg of kaempferol was also investigated. At day 4, parasitemia was determined and percent inhibition was then calculated as described above.

2.9. Determination of Mean Survival Time. The mortality of the experimental mice was monitored daily and the number of days from the time of infection up to death for each mouse in the treatment and control groups throughout the follow-up period was recorded. Mean survival time (MST) was then calculated for each group using the following formula.

$$\text{MST} = \frac{\text{Sum of survival time of all mice in a group}}{\text{Total number of mice in that group}} \quad (3)$$

2.10. Statistical Analysis. Statistical analysis of the data was carried out using GraphPad Prism (GraphPad Prism software, Inc., US). The results are presented as mean ± standard error of mean (SEM). The differences between means of the measured parameters were compared using one-way ANOVA followed by Tukey's post hoc test. The *P* values < 0.05 at 95% confidence were regarded as statistically significant.

3. Results

3.1. Chronic Oral Toxicity of Kaempferol in Mice. Kaempferol was well-tolerated by mice over a period of administration of 30 days. Chronic administration of 2,000 mg/kg of

kaempferol did not result in any overt signs of toxicity. Hepatotoxicity (indicated by AST and ALT), nephrotoxicity (indicated by BUN and creatinine), and hematotoxicity were also ruled out due to a lack of a significant difference with values from control mice (Table 1).

3.2. Suppressive Antimalarial Activity of Kaempferol against Plasmodium berghei ANKA. Kaempferol exerted a significant (*P* < 0.05) dose-dependent chemosuppression against PbANKA (Figure 1). The percentage inhibition at 1, 10, and 20 mg/kg of kaempferol was 16.79%, 31.87%, and 52.89%, respectively. The standard drug, CQ, showed a significant (*P* < 0.001) chemosuppressive effect with 90.11% inhibition, which was higher than the suppression in the kaempferol treated animals. Moreover, a significant (*P* < 0.001) chemosuppression was also observed in a combination treatment of CQ and kaempferol, but the suppression was not statistically different from CQ treated group.

3.3. Prophylactic Antimalarial Activity of Kaempferol against Plasmodium berghei ANKA. To determine the efficacy of kaempferol as an antimalarial prophylactic, mice were administered 1, 10, and 20 mg/kg of kaempferol for 4 consecutive days, while control mice received 10 ml/kg of DW or 10 mg/kg of CQ. A combination treatment was also performed with 10 mg/kg of CQ and 20 mg/kg of kaempferol. Subsequently, these mice were inoculated with PbANKA parasitized erythrocytes of PbANKA by IP injection for 4 days. On day 8, parasitemia was determined using microscopic examination of Giemsa stained thin blood smears. Results (Figure 2) showed that kaempferol possessed significant (*P* < 0.05) chemoprophylactic activity of 24.47% and 40.80% at the doses of 10 and 20 mg/kg, respectively, while the CQ treated showed significant (*P* < 0.001) chemoprophylactic effect with 71.45% inhibition. Moreover, 80.01% inhibition was found in the combination treatment of CQ and kaempferol. However, there was no statistically significant difference between the CQ treated mice with and without kaempferol.

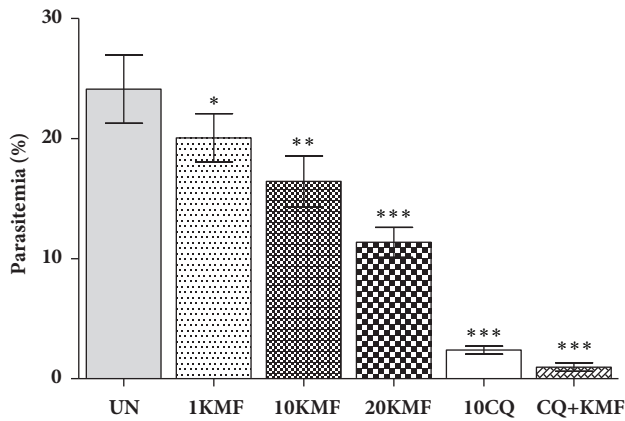


FIGURE 1: Antimalarial effect of kaempferol on PbANKA in a suppressive test. Groups of ICR mice (3 mice per group) were inoculated with 1×10^7 parasitized erythrocytes of PbANKA by IP injection. Two hours later, they were administered with 1, 10, and 20 mg/kg of kaempferol by oral gavage. Untreated control mice received 10 ml/kg of DW while positive control mice were given 10 mg/kg of CQ. A combination treatment of 10 mg/kg of CQ and 20 mg/kg of kaempferol was also administered. The treatment was carried out for 4 consecutive days (days 0-3). On day 4, parasitemia was determined by microscopic examination of Giemsa stained thin blood smears. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$, compared to untreated control. UN; untreated control, 1KMF; 1 mg/kg of kaempferol, 10KMF; 10 mg/kg of kaempferol, 20KMF; 20 mg/kg of kaempferol, and 10CQ; 10 mg/kg of chloroquine. The results are expressed as mean \pm SEM.

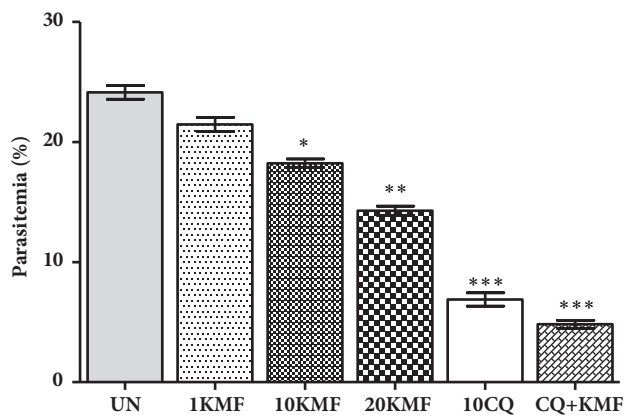


FIGURE 2: Antimalarial effect of kaempferol on PbANKA in a prophylactic test. Groups of ICR mice (3 mice per group) were given 1, 10, and 20 mg/kg of kaempferol by oral gavage for 4 consecutive days (days 0-3). Untreated and positive control mice received 10 ml/kg of DW and 10 mg/kg of CQ, respectively. A combination treatment with 10 mg/kg of CQ and 20 mg/kg of kaempferol was also undertaken. Starting on day 4, the mice were inoculated with 1×10^7 PbANKA parasitized erythrocytes by IP injection for 4 days (days 4-7). On day 8, parasitemia was determined by microscopic examination of Giemsa stained thin blood smears. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$, compared to untreated control. UN; untreated control, 1KMF; 1 mg/kg of kaempferol, 10KMF; 10 mg/kg of kaempferol, 20KMF; 20 mg/kg of kaempferol, and 10CQ; 10 mg/kg of chloroquine. The results are expressed as mean \pm SEM.

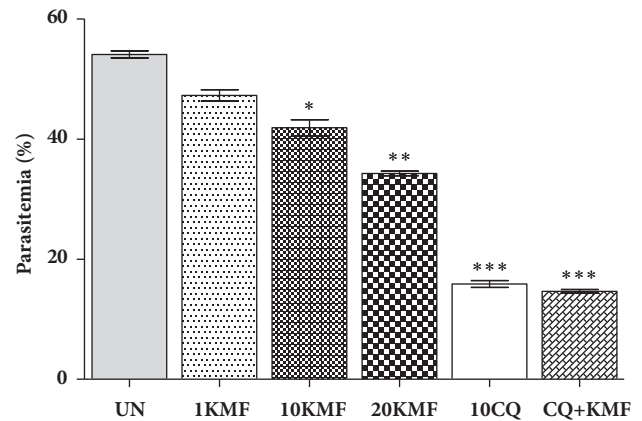


FIGURE 3: Antimalarial effect of kaempferol on PbANKA in a curative test. Groups of ICR mice (3 mice per group) were inoculated with 1×10^7 PbANKA parasitized erythrocytes by IP injection for 4 days (days 0-3). Starting on day 4, the mice were given 1, 10, and 20 mg/kg of kaempferol by oral gavage for 4 consecutive days (day 4-7). Untreated and positive control mice received 10 ml/kg of DW and 10 mg/kg of CQ, respectively. A combination treatment of 10 mg/kg of CQ and 20 mg/kg of kaempferol was also administered. On day 8, parasitemia was determined by microscopic examination of Giemsa stained thin blood smears. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$, compared to untreated control. UN; untreated control, 1KMF; 1 mg/kg of kaempferol, 10KMF; 10 mg/kg of kaempferol, 20KMF; 20 mg/kg of kaempferol, and 10CQ; 10 mg/kg of chloroquine. The results are expressed as mean \pm SEM.

3.4. *Curative Antimalarial Activity of Kaempferol against Plasmodium berghei ANKA.* To assess the curative efficacy of kaempferol mice were inoculated with PbANKA parasitized erythrocytes for 4 consecutive days. On day 4 and for the following 3 days, mice were administered 1, 10, and 20 mg/kg of kaempferol. Control mice received either 10 ml/kg of DW or 10 mg/kg of CQ. A combination treatment was also performed with 10 mg/kg of CQ and 20 mg/kg of kaempferol. Parasitemia was determined on day 8 by examination of Giemsa stained thin blood smears. Results (Figure 3) showed that kaempferol treatment resulted in a significant ($P < 0.05$) reduction in parasitemia, with an inhibition of 22.59% and 36.63% at doses of 10 and 20 mg/kg, respectively. The CQ treated group showed a significant ($P < 0.001$) reduction of parasitemia with 70.64% inhibition, which was similar to the level of inhibition seen in the combination treated mice (72.92%).

3.5. *Effect of Kaempferol on Mean Survival Time of Mice.* The efficacy of kaempferol in mice at all doses, except that of 1 mg/kg, was correlated significantly ($P < 0.05$) with increased mean survival time (MST) compared to the untreated control animals (Table 2). The MST of the CQ treated and combination treated groups were also significantly ($P < 0.001$) increased compared to untreated control animals. In the combination treatment, however, the MST was not significantly different from the CQ treated group.

TABLE 2: Effect of kaempferol on MST of PbANKA infected mice in suppressive, prophylactic, and curative tests.

Tests	Treatments	Doses	Parasitemia (%)	Inhibition (%)	MST (days)
Suppressive	DW	10 ml/kg	24.12	0	9.8 ± 1.5
	Kaempferol	1 mg/kg	20.07	16.79	11.3 ± 1.2
		10 mg/kg	16.43	31.87	16.2 ± 1.2*
	CQ	20 mg/kg	11.36	52.89	24.8 ± 2.5**
		10 mg/kg	2.38	90.11	33.3 ± 2.2* **
CQ + kaempferol	10 mg/kg + 20 mg/kg	0.97	95.98	35.2 ± 2.1* **	
Prophylactic	DW	10 ml/kg	24.14	0	9.5 ± 1.0
	Kaempferol	1 mg/kg	21.48	11.02	11.2 ± 2.1
		10 mg/kg	18.23	24.47	17.3 ± 1.9*
	CQ	20 mg/kg	14.29	40.80	22.2 ± 2.3**
		10 mg/kg	6.89	71.45	26.3 ± 1.5* **
CQ + kaempferol	10 mg/kg + 20 mg/kg	4.83	80.01	29.2 ± 2.1* **	
Curative	DW	10 ml/kg	54.13	0	9.3 ± 1.5
	Kaempferol	1 mg/kg	47.29	12.64	9.7 ± 1.4
		10 mg/kg	41.9	22.59	14.0 ± 1.4*
	CQ	20 mg/kg	24.30	36.63	20.0 ± 1.4**
		10 mg/kg	15.89	70.64	25.7 ± 1.6* **
CQ + kaempferol	10 mg/kg + 20 mg/kg	14.66	72.92	27.2 ± 1.5* **	

* $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$, compared to DW.

4. Discussion

The antimalarial activities of kaempferol, both alone and in combination with CQ against PbANKA infection in mice in suppressive, prophylactic, and curative tests, were investigated. Assessment of the chronic toxicity of kaempferol showed no visible signs of toxicity, hepatotoxicity, nephrotoxicity, hematotoxicity, or death of the mice after oral administration of 2,000 mg/kg of kaempferol daily for 30 days. Hence, the kaempferol can be considered safe according to the OECD guideline which recommends a maximum dose of 2,000 mg/kg for assessing toxicity [13]. The results also showed that kaempferol significantly inhibited PbANKA growth in a dose-dependent manner. The highest activity was observed at a dose of 20 mg/kg with percent inhibition of 52.89%, 40.80%, and 36.63% for chemosuppressive, chemoprophylactic, and curative experiments, respectively. The standard 4-day test is a standard test commonly used for antimalarial screening *in vivo* in which $\geq 30\%$ inhibition in parasitemia following treatment makes a compound to be considered active [14]. The present finding is in agreement with previous research investigating the use of kaempferol for malaria treatment [15]. It has also been reported that kaempferol shows antimalarial activity against chloroquine-resistant *P. falciparum* after 24 h treatment with an IC₅₀ of 106 μM [8]. Therefore, the antimalarial activity of kaempferol might be primarily dependent on its antioxidant and anticancer activities. Oxidative stress resulting from the formation of reactive oxygen species (ROS) plays a critical role in malaria infection. During propagation of the malaria parasite in erythrocytes, toxic by-products that cause hemoglobin

degradation are produced [16]. Additionally, ROS and oxidative stress are also generated by activated monocytes and neutrophils during infection [17, 18]. It has also been shown that the malaria parasite causes cell membrane injury to both infected and uninfected erythrocytes through lipid peroxidation [19, 20]. As a flavonoid, kaempferol is able to inhibit oxidative stress, ROS formation, and lipid peroxidation [21–23], and there is a correlation between its antioxidant and antimalarial activities [4]. In addition, inhibition of glycogen synthase kinase-3 β (GSK3 β) of the malaria parasite has been reported to be a molecular basis of the antimalarial effect of kaempferol [24]. GSK3 has been described to play a critical role in the host response to malaria infection [25]. Therefore, the antimalarial effects of kaempferol in the present study might be mediated through the inhibition of GSK3 β .

Kaempferol has been described as having anticancer properties by inducing apoptosis through the caspase cascade and the MAPK pathway [26–28], which is similar to artemisinin, an effective antimalarial drug currently in use, that also has been reported to have anticancer and related potent antimalarial activities [29]. Hence, it can be hypothesized that the antioxidant and anticancer properties of kaempferol may be responsible for the antimalarial activity observed in the present study.

MST is another parameter for evaluating the antimalarial activity of test compounds. Accordingly, test compounds that result in survival time greater than that of the untreated group are considered as active [30, 31]. This study showed that infected mice treated with 10 and 20 mg/kg of kaempferol lived significantly longer than animals in the untreated group. However, the sample sizes were small; it does allow

researchers to focus on really big effect sizes. These findings provide a basis for further investigations of kaempferol as a potential candidate compound for antimalarial drug development.

5. Conclusions

Oral administration of kaempferol at the highest dose of 2,000 mg/kg induced no adverse effects, signifying the safety of this compound in mice. Kaempferol both alone and in combination with CQ exerted a reasonable antimalarial activity and significantly prolonged the survival time of PbANKA infected mice. A dose of 20 mg/kg of kaempferol was observed to have the strongest antimalarial activity, particularly in chemosuppression. However, these findings are only preliminary and thus further studies investigating the mechanism by which kaempferol exert its antimalarial activity thereby resulting in prolonged MST in PbANKA infected mice are recommended.

Data Availability

The Graph data used to support the findings of this study have been deposited in the figshare (DOI: 10.6084/m9.figshare.6981866 or <https://figshare.com/s/4289e1f86751ca8ffd49>).

Conflicts of Interest

The authors have declared that there are no conflicts of interest regarding the publication of this manuscript.

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