

In vivo antimalarial activity of *Cyperus rotundus* and its combination with dihydroartemisinin against *Plasmodium berghei*

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Abstract

Background

The increase in the number of drug-resistant *Plasmodium* species continues to be a serious public health concern. Therefore, identification of potential novel antimalarial drugs derived from therapeutic plants could help solve this issue. This study investigated whether *Cyperus rotundus* aqueous crude extract (CRE) and its combination with dihydroartemisinin (DHA) were effective against *Plasmodium berghei* ANKA-infected mice.

Methods

CRE was prepared from *C. rotundus* rhizomes and evaluated in acute and subacute toxicity tests on BALB/c mice. The antimalarial effectiveness of CRE was assessed at 100, 200, and 400 mg/kg in a 4-day suppressive test with curative and prophylactic testing and measurement of packed cell volume (PCV), body weight (BW), rectal temperature, and mean survival time (MST).

Results

Following acute and subacute treatment, CRE caused no harmful effects or mortality in mice. When compared with that in the untreated control, infected mice administered with 400 mg/kg of CRE in a 4-day suppressive test exhibited the strongest antimalarial activity (55.30% inhibition) with prolonged MST. However, curative and prophylactic assays did not reveal CRE to have antimalarial activity. In comparison with that achieved with the single therapy, the combination of DHA and CRE at ED_{50/2} (1 and 200 mg/kg, respectively) produced considerable antimalarial activity at 90.08% inhibition with synergism (combination index = 0.21701). For the other parameters, CRE administration prevented malarial-induced changes in PCV, BW, and rectal temperature.

Conclusions

CRE treatment significantly inhibited malaria in the 4-day suppressive test, and CRE combined with DHA had a synergistic antimalarial effect.

1. Background

Malaria remains one of the most serious diseases and is a significant public health concern. Malaria is caused by a *Plasmodium* parasite that is transmitted by female *Anopheles* mosquitoes, and five *Plasmodium* species can infect humans: *P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae*, and *P. knowlesi* [1]. In 2021, an estimated 247 million cases of malaria and 619,000 deaths were reported in 84 malaria-

endemic nations with children under the age of 5 and pregnant women constituting high-risk groups [2]. Even though an effective malaria vaccine is the most effective long-term control option, current research on vaccine development remains at the preclinical phase. Thus, the malaria control strategy mainly focuses on the use of antimalarial medications, although *Plasmodium* strains resistant to antimalarial drugs have rapidly emerged [3]. The World Health Organization recommends artemisinin-based combination therapy (ACT) as the initial treatment in malaria-endemic countries. Nonetheless, artemisinin-resistant parasites have been reported to be spreading in the Greater Mekong Subregion of Southeast Asia over the past decade [3–5]. This has driven the creation of antimalarial drugs with novel mechanisms of action, and medicinal plants have become regarded as potential agents that could be used in ACT. However, the claimed efficacy of medicinal plants must be scientifically evaluated and toxicity assays conducted.

The nutgrass, *Cyperus rotundus* L., a sedge of the Cyperaceae family, is a colonial and perennial herb cultivated in India and commonly used as in Asian countries to cure stomach and bowel ailments, as well as inflammatory conditions. Several studies have documented that *C. rotundus* extract has pharmacological properties, including antioxidant, anti-inflammatory, antipyretic, antidiarrheal, antiarthritic, antihyperglycemic, antihyperlipidemic, antiulcer, antimicrobial, antihelminthic, antiallergic, neuroprotective, gastroprotective, hepatoprotective, wound healing, and analgesic effects [6, 7]. The phytochemical components of *C. rotundus* include alkaloids, flavonoids, phenols, terpenoids, glycosides, tannins, saponin, steroids, starch, and a large number of novel sesquiterpenoids [8]. In addition, *C. rotundus* extract has demonstrated potent antimalarial activity against *P. falciparum* in culture [9, 10]. However, the antimalarial activity of *C. rotundus* alone and in combination therapy with artemisinin derivatives have not yet been investigated in animal models. Hence, this study sought to evaluate the efficacy of aqueous crude extract of *C. rotundus* and its combination with dihydroartemisinin against mice infected with *P. berghei*.

2. Methods

2.1 Plant material and extraction

Fresh *C. rotundus* rhizomes were gathered in the Chiang Mai province of Thailand in October 2022. Botanical identification was performed, and a voucher specimen was stored at the Research Institute for Health Sciences, Chiang Mai University, Chiang Mai, Thailand. Plant materials were washed with water and air-dried at room temperature. Dried material was then reduced to coarse powder and subsequently prepared as a fine powder using an electric blender. For aqueous crude extraction, 30 g of dried powdered plant material was extracted in 150 mL of boiled distilled water (DW) at room temperature for 20–24 h using an orbital shaker and filtered through a Whatman no.1 filter paper. The filtrate was then centrifuged at 4,000 *g* for 10 min, and supernatant was collected. The supernatant was lyophilized to obtain aqueous crude extract of *C. rotundus* (CRE), which was stored at 4°C [11]. CRE was dissolved in DW at chosen doses for oral administration by gavage to mice.

2.2 Preparation of dihydroartemisinin

DHA was purchased from Sigma-Aldrich (Sigma-Aldrich, Inc., St. Louis, MO, USA). Fresh doses of DHA were prepared in DW and stored at 4°C.

2.3 Experimental mice

Four-week-old male BALB/c mice (22–25 g) were purchased from Nomura Siam International, Thailand. Mice were kept for a minimum of one week in a standard laboratory environment and were given standard pellets (CP diet 082, Perfect Companion Company, Thailand) and access to clean water *ad libitum*. The National Institutes of Health Guidelines for Care and Use of Laboratory Animals were followed for all mouse-related experiments (2011). Walailak University Animal Care and Use Committee approved all experimental protocols (WU-ACUC-65077).

2.4 Acute toxicity testing

The acute toxicity of the CRE was determined in accordance with the Organization for Economic Cooperation and Development (OECD) Guideline 423 [12]. BALB/c mice were randomly divided into two groups ($n = 5$). The first group received a single dose of 2,000 mg/kg of CRE orally by gavage, whereas the second group received DW. During the first 30 min and daily thereafter, mice were observed for any clinical signs of toxicity or mortality.

2.5 Subacute toxicity testing

The procedures described in Guide 407 of the OECD guidelines was performed using the limit test with 1,000 mg/kg of CRE [13]. BALB/c mice ($n = 5$) were administered with a daily dose of 1,000 mg/kg of CRE for orally by gavage for 30 days. The control group received 10 mL/kg of DW. At the end of the 30-day observation period, the mice were anesthetized, and blood was then collected by cardiac puncture in heparinized vacuum tubes for biochemical marker measurement.

2.6 Biochemical marker measurement

After centrifuging blood samples at 3,000 *g* for 10 min, plasma was collected for biochemical marker analysis. Using an automated clinical chemistry analyzer (Cobas c111), the levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), blood urea nitrogen (BUN), and creatinine were measured.

2.7 Rodent malaria parasite

P. berghei ANKA strain (PbANKA) obtained from the Malaria Research and Reference Reagent Resource Center was used in this study. Parasites were maintained in mice by sequential blood passage through intraperitoneal (IP) injection with a 5-day interval. Infected mouse blood with parasitemia of 15–30% was collected via cardiac puncture and diluted with normal saline to 5×10^7 parasitized erythrocytes per mL. Each mouse was then inoculated with 0.2 mL of this diluted blood via IP injection.

2.8 Determination of parasitemia

Parasitemia was determined via microscopic examination. Thin blood smears taken from the tail blood of PbANKA-infected mice were prepared on microscopic slides. Smears were fixed with absolute methanol and stained with 10% Giemsa dye solution. Parasitized erythrocytes were identified under a light microscope with 100× oil immersion lens. Parasitemia was calculated using:

$$\%parasitemia = \frac{Numberofparasitizederythrocytes}{Totalnumberoferythrocytes} \times 100$$

2.9 Determination of mean survival time

During the follow-up period, the daily mouse mortality and the number of days from the time of inoculation until death was recorded, up to a maximum of 30 days. The mean survival time (MST) was calculated using:

$$MST = \frac{Sumofsurvivaltimeofallmiceinagroup}{Totalnumberofmiceinthatgroup}$$

2.10 Determination of packed cell volume

The packed cell volume (PCV) was used to investigate the efficacy of CRE and in combination with DHA in preventing hemolysis caused by malaria infection. Tail blood was placed in heparinized hematocrit tubes and centrifuged for 5 min at 12,000 rpm. PCV is a measurement of the proportion of erythrocytes to plasma in whole blood, and was calculated using:

$$\%PCV = \frac{Volumeoftotalerythrocytesinagivenvolumeofblood}{Totalbloodvolume} \times 100$$

2.11 Determination of body weight and rectal temperature

The body weight (BW) of each mouse was measured using a digital balance, and the rectal temperature was measured using a digital thermometer.

2.12 Four-day suppressive test

The antimalarial activity on early infection of CRE was evaluated by the Peter's 4-day suppressive test against PbANKA infection in mice [14]. Twenty-five BALB/c mice were randomly divided into five groups (n = 5 per group) and inoculated with 1×10^7 parasitized erythrocytes of PbANKA via IP injection. At 2 h after infection, mice were orally administered by gavage with 100, 200, and 400 mg/kg of CRE. Untreated and positive groups were administered with 10 mL/kg of DW and 5 mg/kg of DHA, respectively. Treatment was performed once a day for four consecutive days (D0–D3). On D4, parasitemia, PCV, BW, rectal temperature, and MST were measured. In addition, percent inhibition was calculated using:

$$\%inhibition = \frac{(parasitemiaoftheuntreatedgroup - parasitemiaoftestedgroup)}{parasitemiaoftheuntreatedgroup} \times 100$$

2.13 Rane's (curative) test

The curative activity was assessed using the method defined by Ryley and Peters 1970 [15]. On D0, 25 BALB/c mice were inoculated via IP injection with 1×10^7 PbANKA-parasitized erythrocytes. On D4, the groups were treated with CRE (100, 200, and 400 mg/kg) orally by gavage, whereas untreated and positive groups received 10 mL/kg of DW and 5 mg/kg of DHA, respectively. Treatment was performed once a day for 4 consecutive days (D4–D7). On D8, parasitemia, PCV, BW, rectal temperature, and MST were then measured. Percent inhibition was also calculated.

2.14 Prophylactic test

The method described by Peters (1965) was used to assess the prophylactic activity [16]. Twenty-two BALB/c mice were administered with 100, 200, and 400 mg/kg of CRE orally by gavage once a day for four consecutive day (D0–D3). Mice were then inoculated with 1×10^7 PbANKA-parasitized erythrocytes via IP injection on D4 and monitored for 72 h (D4–D7). Untreated and positive controls received 10 mL/kg of DW and 5 mg/kg of DHA, respectively. On D8, parasitemia, PCV, BW, rectal temperature, and MST were measured, and percent inhibition was calculated.

2.15 Evaluation of effective dose

The standard 4-day suppressive test was used to determine the effective dose (ED_{50}) of the drugs (CRE and DHA) [14]. BALB/c mice ($n = 5$) were injected intraperitoneally with 1×10^7 parasitized erythrocytes of PbANKA. At 2 h after infection, mice were orally administered with CRE (50, 100, 200, 400, and 600 mg/kg) and DHA (0.1, 1, 5, 10, and 20 mg/kg) daily for four days (D0–D3). Infected mice without treatment received 10 mL/kg of DW. On day 4, the parasitemia was measured and percent inhibition was calculated. Using a nonlinear regression for the dose-response variable slope, the optimal ED_{50} value was determined.

2.16 Combination antimalarial treatment

ED_{50} values of CRE and DHA was used to evaluate combination treatment. The CRE and DHA were combined at fixed ratio (1:1) of ED_{50} , $ED_{50/2}$, $ED_{50/4}$, and $ED_{50/8}$. The combination was tested using the standard 4-day suppressive test [14]. On D4, parasitemia was measured and percent inhibition calculated. Points above the joint line indicated synergism while those around the line or below indicated additive or antagonistic interactions, respectively. Combination index (CI) value was also performed.

2.17 Statistics

Results are displayed as mean $\pm$ standard error of mean (SEM). Using a nonlinear regression for the dose-response variable slope, the optimal ED_{50} value was determined. One-way ANOVA with Tukey's post hoc test was used to compare means of control and treatment groups. Significance was considered with 95%

confidence intervals and $p < 0.05$. Data were analyzed with GraphPad Prism 9.5.0 (GraphPad Software, Inc., San Diego, CA, USA). CompuSyn software (ComboSyn, Inc., USA) was used to calculate the CI value to indicate synergism ($CI < 1$), additive effect ($CI = 1$), and antagonism ($CI > 1$).

3. Results

3.1 Toxicity test of CRE in mice

The acute toxicity test revealed that oral administration of CRE at a single dose of 2,000 mg/kg in mice did not cause mortality or produce any signs of toxicity in treated mice, and all mice exhibited normal behavior throughout the 14-day observation period. In the subacute toxicity test, administration with 1,000 mg/kg of CRE daily for 30 days did not induce any toxic symptoms or cause mortality in mice, which behaved normally throughout the experiment. No significant changes in the plasma levels of AST, ALT, ALP, BUN, or creatinine were observed in biochemical tests of liver or renal function (Table 1). Consequently, the LD₅₀ value for CRE was > 2,000 mg/kg, and CRE was subsequently considered safe for oral administration.

Table 1
Effect of oral administration of CRE on biochemical markers in mice

Biochemical markers	Control	CRE (1,000 mg/kg)
AST (U/L)	98.83 ± 3.04	94.30 ± 1.40
ALT (U/L)	52.65 ± 1.93	50.40 ± 1.16
ALP (U/L)	152.33 ± 2.38	154.40 ± 1.69
BUN (mg/dl)	18.80 ± 0.63	18.23 ± 0.21
Creatinine (mg/dl)	0.52 ± 0.07	0.51 ± 0.04

Results were expressed as mean ± SEM (n = 5).

3.2 Propagation of PbANKA infection in mice

At day 12 after infection, parasitemia reached 45.5 ± 2.7%, whereas the PCV decreased from 53.2 ± 3.5% to 20.4 ± 3.1% (Fig. 1a). In addition, BW and rectal temperature were considerably decreased in PbANKA-infected mice (Fig. 1b), and all infected mice died within two weeks (Fig. 1c). Hence, PbANKA infection induced malaria-associated hemolysis, BW loss, and hypothermia.

3.3 Suppressive antimalarial effect of CRE on PbANKA-infected mice

CRE at a dose of 400 mg/kg significantly ($p < 0.01$) inhibited parasitemia by 55.3% compared with that in the untreated control, whereas DHA at a dose of 5 mg/kg inhibited parasitemia by 90.1% (Fig. 2a). DHA-

treated mice exhibited a significant ($p < 0.05$) decrease in PCV ($43.7 \pm 0.8\%$) compared with that in the healthy control. However, mice treated with 400 mg/kg of CRE did not demonstrate a significant reduction in PCV ($49.9 \pm 0.7\%$) compared with that in the healthy control ($52.6 \pm 1.2\%$) (Fig. 2b). In addition, mice treated with 400 mg/kg of CRE did not exhibit a significant decrease in BW (24.0 ± 0.7 g) or rectal temperature ($35.3 \pm 0.9^\circ\text{C}$) when compared with these parameters in the healthy control (25.3 ± 1.2 g and $37.2 \pm 0.5^\circ\text{C}$, respectively) (Fig. 2c–2d). CRE (400 mg/kg) did significantly ($p < 0.001$) prolong the MST (26.0 ± 3.2 days) of infected mice relative to that of the untreated control (10.4 ± 3.3 days) (Fig. 2e). However, mice treated with 100 and 200 mg/kg of CRE exhibited significant ($p < 0.05$) decreases in PCV, BW, and rectal temperature (Fig. 2b–2e). With prolonged MST, 400 mg/kg of CRE exerted chemosuppressive antimalarial activity and protective effects on PCV reduction, BW loss, and rectal temperature decrease.

3.4 Curative antimalarial effect of CRE in PbANKA-infected mice

The parasitemia level did not significantly decrease at any of the three doses of CRE used to treat mice (Fig. 3a). Compared with that in the healthy control group, mice administered with 400 mg/kg of CRE did not exhibit significant reductions in PCV ($48.8 \pm 0.7\%$), BW (23.6 ± 0.8 g), or rectal temperature ($34.2 \pm 0.6^\circ\text{C}$), whereas significant ($p < 0.01$) PCV reduction ($37.5 \pm 0.9\%$) was observed in the DHA-treated group (Fig. 3b–3d). Moreover, the MST (20.4 ± 3.6 days) of mice administered 400 mg/kg of CRE was longer than that of the untreated mice (10.0 ± 3.2 days) (Fig. 3e).

3.5 Prophylactic antimalarial effect of CRE on PbANKA-infected mice

CRE lacked chemoprophylactic activity at all three doses in PbANKA-infected mice (Fig. 4a). Compared with that in the untreated control, only the 400 mg/kg CRE-treated group demonstrated a protective effect on PCV ($49.4 \pm 0.7\%$), BW (23.8 ± 0.6 g), and rectal temperature reduction ($35.5 \pm 0.4^\circ\text{C}$) (Fig. 4b–4d). PCV remained significantly ($p < 0.01$) reduced in the DHA-treated group ($41.5 \pm 0.9\%$) (Fig. 4b). In addition, mice treated with CRE at a dose of 400 mg/kg lived significantly ($p < 0.05$) longer than their untreated counterparts (17.6 ± 0.9 and 9.6 ± 2.6 days, respectively) (Fig. 4e). Therefore, prophylactic antimalarial activity was not observed in mice treated with all three doses of CRE, although CRE at 400 mg/kg protected against PCV reduction, BW loss, rectal temperature decrease, and prolonged MST.

3.6 Combination treatment of DHA and CRE in PbANKA-infected mice

The ED_{50} values of DHA and CRE were determined to be 2.1 ± 0.3 and 400.4 ± 0.2 mg/kg, respectively, on the basis of the dose-response curve (Fig. 5). The combination of DHA and CRE at ED_{50} and $\text{ED}_{50/2}$ exhibited significant ($p < 0.001$) antimalarial activity compared with that of the untreated control with 74.83% and 90.08% inhibition of parasites, respectively (Fig. 6a). Interestingly, DHA combined with CRE at

a dose of ED_{50/2} demonstrated significantly ($p < 0.01$) more potent antimalarial activity than either DHA or CRE alone. In addition, synergistic interaction of the combination treatment at the ED₅₀ and ED_{50/2} doses (CI = 0.92488 and 0.21701, respectively) was observed as indicated by CI value of < 1.0 (Table 2) and the interaction points above the joint line (Fig. 6b). Nonetheless, the ED_{50/4} and ED_{50/8} (CI = 1.85871 and 2.34981, respectively) of DHA and CRE combination demonstrated antagonistic interaction.

Table 2
Combination index of interaction between DHA and CRE
against PbANKA infection in mice

Antimalarial test		Dose (mg/kg)		CI value
		DHA	CRE	
Combination	ED ₅₀	2	400	0.92488 ^a
	ED _{50/2}	1	200	0.21701 ^a
	ED _{50/4}	0.5	100	1.85871 ^b
	ED _{50/8}	0.25	50	2.34981 ^b
^a CI < 1; synergism, ^b CI > 1; antagonism				

4. Discussion

Recent resistance to artemisinin and its derivatives has emerged in Southeast Asia. Consequently, new antimalarial drug candidate, such as those derived from medicinal plants, are urgently required. This study therefore investigated the antimalarial activity of CRE and its combination with DHA against PbANKA infection in mice. The acute toxicity test in mice evaluates the adverse effects over a short period following administration of a single, high dose of a substance. No signs of toxicity, behavioral change, or mortality were observed in mice administered with 2,000 mg/kg of CRE. Thus, CRE with an LD₅₀ > 2,000 mg/kg can be considered nontoxic. Toxicity evaluation after repeated dosing provides evidence of dose response with potential health risks after 30 days of subacute toxicity testing. Here, dosing of mice at 1,000 mg/kg of CRE for 30 days did not produce any abnormal signs, toxicity, or death or any changes in biochemical markers. After CRE treatment, the plasma levels of AST, ALT, ALP, BUN, and creatinine levels remained within physiological limits, indicating that CRE did not adversely affect the liver or kidneys. Thus, CRE can be regarded as relatively harmless in terms of acute and subacute toxicity. If the LD₅₀ of the test substance is > 3-fold the minimum effective dose (400 mg/kg), the drug may be an appropriate candidate for further study [17].

During ongoing PbANKA infection in mice, malaria-associated hemolysis, BW loss, and a decrease in rectal temperature were observed. PCV reduction is a defining characteristic of both human and rodent malaria. Mice with a high parasitemia experience a rapid destruction of parasitized and uninfected

erythrocytes, suppression of erythropoietin, and dyserythropoiesis, and may develop severe anemia [18, 19]. Additionally, an increase in the amount of free radicals and inflammatory cytokines, followed by oxidative stress and lipid peroxidation, may contribute to hemolysis during malaria infection [20]. BW loss is a common symptom of *P. berghei* infection in mice and is associated with an increase in parasitemia. BW loss may be caused by a catabolic action on stored lipids, a hypoglycemic effect of the parasite, or an anorexigenic effect on mouse appetite that suppresses food consumption [21, 22]. Mice infected with *P. berghei* experienced a decline in rectal temperature because of the presence of the parasite. Malarial infections can decrease the metabolic rate to cause a decrease in rectal temperature. Therefore, these results agreed with those of other studies [23–25].

The antimalarial activity of CRE was investigated using the 4-day suppressive, curative, and prophylactic tests. PbANKA-infected mice were evaluated for schizontocidal activity against early, established, and residual infections. In the 4-day suppressive test, CRE significantly reduced the level of parasitemia, and the highest parasite inhibition (55.3%) was found at 400 mg/kg. Thus, CRE may exert an antimalarial activity that potentially mitigated the early infection by PbANKA. General extracts that inhibit parasitemia by $\geq 30\%$ are considered active [26]. *In vivo* antimalarial activity can be classified as moderate, good, or very good if the percentage of inhibition is $\geq 50\%$ at doses of 500, 250, and 100 mg/kg, respectively [27]. Consequently, this study showed that CRE possesses active and moderate 4-day suppressive antimalarial activity. The antimalarial activity of crude extracts of medicinal plants is due to the presence of active compounds. Several studies have implicated secondary metabolites, such as flavonoids, phenols, terpenoids, alkaloids, saponins, tannins, glycosides, and anthraquinone, in this antimalarial activity [28–30]. Phytochemical analysis has revealed that CRE contains alkaloids, flavonoids, phenols, terpenoids, glycosides, tannins, saponin, steroids, starch, and many novel sesquiterpenoids [8]. α -Cyperone and β -selinene autoxidation products derived from *C. rotundus* are highly promising antimalarial compounds [31]. Furthermore, sesquiterpenoids, such as patchoulone, caryophyllene α -oxide, 10,12-peroxycalamenene, and 4,7-dimethyl-1-tetralone, have been reported to exhibit antimalarial activity against *P. falciparum* in culture [10]. Therefore, the antimalarial activity observed in the CRE may be attributable to such secondary active compounds, which may act singly or in combination. However, it is necessary to identify the compounds responsible for the parasite inhibition. The antimalarial activity of CRE might be mediated by various mechanisms, including the response of the antioxidant and immunomodulatory systems, suppression of protein synthesis suppression, inhibition of enzyme activity, interference with erythrocyte invasion by parasites, inhibition of the parasite growth and multiplication, blocked entry of nutrients into the parasite, inhibition of heme polymerization, or other unidentified mechanisms [30].

Parasite inhibition was not observed at the lower doses (100 and 200 mg/kg) of CRE. This might be because of the active compounds in the extract were only present at a low level, and their activity may not be detectable at these doses. In both curative and prophylactic studies, no significant differences in inhibition of parasitemia by CRE at any of the three doses were observed compared with that of the untreated control. These results suggest that CRE has a greater chemosuppressive effect on early infection than on either established or residual infections. This lack of effect may be associated with the

metabolic processing of CRE following administration and the reduction of its concentration in the body and could also be related to the rapid multiplication of the parasite in an established infection, where the parasite is growing exponentially. Additionally, the absence of effect of low concentrations may be due to the rapid hepatic metabolism or metabolic inactivation and clearance of the CRE active compounds before parasite inoculation in the prophylactic test.

PbANKA infection is typically manifested by a decrease in PCV, loss of BW, and decline in rectal temperature, and treatment with CRE with antimalarial activity could therefore protect against these abnormalities. In all test models, the highest CRE dose (400 mg/kg) significantly ($p < 0.01$) inhibited the reduction of PCV, BW, and rectal temperature compared with those in untreated controls. CRE treatment could ameliorate anemia by preventing the destruction of erythrocytes caused by PbANKA. The antioxidant flavonoids and tannins in CRE may significantly help to protect erythrocytes from oxidative stress and inflammation during infection [32]. In addition, the polyphenolic compounds in the CRE may increase the survival rate of both uninfected and infected erythrocytes [8]. Surprisingly, a significant ($p < 0.01$) decrease in PCV was observed in all tested models in mice treated with DHA. Artemisinin drugs and derivatives are known to kill malaria parasites by inducing oxidative stress following the activation of the peroxide bridge, which generates reactive metabolites that cause hemolysis [33]. Additionally, artemisinin-induced and post-artemisinin-delayed hemolysis have been reported [34, 35]. This finding is consistent with other reports on artemisinin-induced hemolysis in PbANKA-infected mice [36–38]. The mechanism whereby CRE prevents BW loss may involve the reduction of parasitemia in PbANKA-infected mice to enable normal metabolism and growth to continue unimpeded. In addition, this is probably due to the activation of an appetite stimulant and the addition of vitamin B [30]. CRE treatment stabilized temperatures in PbANKA-infected mice. This may be due to the CRE suppressing parasites and regulating pathological and immune processes in infected mice, thereby compensating for the decrease in metabolic rate that causes a decrease in rectal temperature. Furthermore, the presence of active metabolites, including polyphenols, flavonoids, terpenoids, steroids, tannins, glycosides, and saponins, which tend to stabilize temperature, may prevent the PbANKA-induced decrease in rectal temperature [3]. However, activity of 100 and 200 mg/kg of CRE was insufficient to prevent these abnormalities in PbANKA-infected mice.

MST is additional criterion used to evaluate the antimalarial activity of plant extracts, and extracts that produce a longer MST than that of the untreated control are considered active. In this study, PbANKA-infected mice treated with 400 mg/kg of CRE lived significantly longer than untreated controls in all three antimalarial test models. This may be due to the antimalarial properties of the CRE and the prevention of PCV depletion, BW loss, and decrease in rectal temperature. However, the MST of mice treated with CRE was shorter than that of mice treated with DHA. The current findings were consistent with those of previous studies [23, 37, 38].

Combination strategies are preferred over single strategies in treating malaria, and we therefore tested the combination of CRE and DHA on PbANKA-infected mice. The combination of CRE and DHA at doses of $ED_{50/1}$ and $ED_{50/2}$ showed significant antimalarial activity compared with that of the untreated control,

although only ED_{50/2} exerted a significant effect compared with that of the CRE or DHA treatment alone. As indicated by the CI value < 1.0, a synergistic interaction was observed. However, the lower doses (ED_{50/4} and ED_{50/8}) of the combination had no significant effect on parasitemia. The antimalarial mechanism for this combination treatment is not readily apparent from our current research as both compounds could exert an antimalarial synergistic effect via their own individual actions or via a novel pathway induced by the combination. Therefore, we consider that the combination of CRE and DHA may provide an alternative antimalarial therapeutic approach.

5. Conclusions

Our study clearly indicates that CRE has potent antimalarial activity with the highest parasitemia inhibition observed at 400 mg/kg in the 4-day suppressive test. Combining CRE with DHA at the doses of ED_{50/2} showed more successful chemotherapeutic treatment in the PbANKA-infected mouse model. Additionally, CRE in combination with DHA could inhibit PCV reduction, BW loss, and hypothermia induced by malaria infection and prolong MST. These findings also support the traditional claim of *C. rotundus* for malarial treatment. However, further investigations should be conducted to isolate and identify the active compounds and elucidate the mechanism of action for antimalarial activity from this extract.

Abbreviations

ACT: artemisinin-based combination therapy

ALP: alkaline phosphatase

ALT: alanine aminotransferase

ANOVA: analysis of variance

AST: aspartate aminotransferase

BUN: blood urea nitrogen

BW: body weight

CI: combination index

CO: combination treatment

CRE: aqueous crude extract of *Cyperus rotundus*

DHA: dihydroartemisinin

DW: distilled water

ED: effective dose

H: healthy control

MST: mean survival time

PbANKA: *Plasmodium berghei* ANKA strain

PCV: packed cell volume

SEM: standard error of mean

UN: untreated control

Declarations

Ethics approval and consent to participate

All experimental protocols involving animal were reviewed and approved by Walailak University Animal Care and Use Committee (protocol number WU-ACUC-65077) based on the Code of Practice for the Care and Use of Animals for Scientific Purposes, National Committee for Research Animal Development, National Research Council of Thailand. All methods were performed in accordance with the relevant guidelines and regulations (The National Research Council's Guide for the Care and Use of Laboratory Animals).

Consent for publication

Not applicable

Availability of data and materials

The data supporting the findings of this study are openly available on Figshare at <https://figshare.com/s/ef64c9a087997c969694> (DOI: 10.6084/m9.figshare.22211587). Materials will be made available on request.

Competing interests

The authors declare no competing interests.

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Authors' contributions

Sakaewan Ounjaijean: Methodology, Formal analysis, Investigation, Resources, Visualization, Data curation, Writing – Review & Editing. **Charupa Lektip:** Data curation, Writing – Review & Editing. **Voravuth Somsak:** Conceptualization, Methodology, Validation, Writing – Original Draft, Supervision. All authors read and approved the final manuscript.

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References

1. White NJ, Pukrittayakamee S, Hien TT, Faiz MA, Mokuolu OA, Dondorp AM. Malaria. *Lancet*. 2014;383:723-35.
2. WHO: World Health Organization. World malaria report. 2022. <https://www.who.int/teams/global-malaria-programme/reports/world-malaria-report-2022>. Accessed 03 April 2023.
3. Tang YQ, Ye Q, Huang H, Zheng WY. An overview of available antimalarials: discovery, mode of action and drug resistance. *Curr Mol Med*. 2020;20:583-92.
4. Hassett MR, Roepe PD. Origin and spread of evolving artemisinin-resistant *Plasmodium falciparum* malarial parasites in Southeast Asia. *Am J Trop Med Hyg*. 2019;101:1204-11.
5. Imwong M, Suwannasin K, Kunasol C, Sutawong K, Mayxay M, Rekol H, et al. The spread of artemisinin-resistant *Plasmodium falciparum* in the Greater Mekong subregion: a molecular epidemiology observational study. *Lancet Infect Dis*. 2017;17:491-7.
6. Taheri Y, Herrera-Bravo J, Huala L, Salazar LA, Sharifi-Rad J, Akram M, et al. *Cyperus* spp.: a review on phytochemical composition, biological activity, and health-promoting effects. *Oxid Med Cell Longev*. 2021;2021:4014867.
7. Wang F, Zhang S, Zhang J, Yuan F. Systematic review of ethnomedicine, phytochemistry, and pharmacology of *Cyperus rhizoma*. *Front Pharmacol*. 2022;13:965902.
8. Pirzada AM, Ali HH, Naeem M, Latif M, Bukhari AH, Tanveer A. *Cyperus rotundus* L.: traditional uses, phytochemistry, and pharmacological activities. *J Ethnopharmacol*. 2015;174:540-60.
9. Kaushik NK, Bagavan A, Rahuman AA, Mohanakrishnan D, Kamaraj C, Elango G, et al. Antiplasmodial potential of selected medicinal plants from eastern Ghats of South India. *Exp Parasitol*. 2013;134:26-32.
10. Thebtaranonth C, Thebtaranonth Y, Wanauppathamkul S, Yuthavong Y. Antimalarial sesquiterpenes from tubers of *Cyperus rotundus*: structure of 10,12-peroxycalamenene, a sesquiterpene endoperoxide. *Phytochemistry*. 1995;40:125-8.

11. Mohamed AI, Beseni BK, Msomi NZ, Salau VF, Erukainure OL, Aljoundi A, et al. The antioxidant and antidiabetic potentials of polyphenolic-rich extracts of *Cyperus rotundus* (Linn.). J Biomol Struct Dyn. 2022;40:12075-87.
12. OECD Guidelines for Testing of Chemicals: Acute Oral Toxicity—Acute Toxic Class Method. Test No. 423, Adopted 22 March 1996, and Revised Method Adopted 17 December 2001. Paris: OECD; 2001.
13. OECD Guideline for Testing of Chemicals. Repeated Dose 28-Day Oral Toxicity in Rodents, Test No. 407. Paris: OECD; 2008.
14. Peters W. The chemotherapy of rodent malaria, XXII. The value of drug-resistant strains of *P. berghei* in screening for blood schizontocidal activity. Ann Trop Med Parasitol. 1975;69:155-71.
15. Ryley JF, Peters W. The antimalarial activity of some quinolone esters. Ann Trop Med Parasitol. 1970;64:209-22.
16. Peters W. Drug resistance in *Plasmodium berghei* Vincke and Lips, 1948. I. Chloroquine resistance. Exp Parasitol. 1965;17:80-9.
17. Plirat W, Chaniad P, Phuwajaroanpong A, Septama AW, Punsawad C. Phytochemical, antimalarial, and acute oral toxicity properties of selected crude extracts of Prabchompoothaweep remedy in *Plasmodium berghei*-infected mice. Trop Med Infect Dis. 2022;7:395.
18. Lamikanra AA, Brown D, Potocnik A, Casals-Pascual C, Langhorne J, Roberts DJ. Malarial anemia: of mice and men. Blood. 2007;110:18-28.
19. Jauréguiberry S, Ndour PA, Thellier M, Caumes E, Buffet P. Patterns of anemia during malaria. Int J Infect Dis. 2015;33:89.
20. Mohandas N, An X. Malaria and human red blood cells. Med Microbiol Immunol. 2012;201:593-8.
21. Ounjaijean S, Romyasamit C, Somsak V. Evaluation of antimalarial potential of aqueous crude *Gymnema inodorum* leaf extract against *Plasmodium berghei* infection in mice. Evid Based Complement Alternat Med. 2021;2021:9932891.
22. Chaniad P, Techarang T, Phuwajaroanpong A, Punsawad C. Antimalarial activity and toxicological assessment of *Betula alnoides* extract against *Plasmodium berghei* infections in mice. Evid Based Complement Alternat Med. 2019;2019:2324679.
23. Ounjaijean S, Somsak V. Effect of allicin and artesunate combination treatment on experimental mice infected with *Plasmodium berghei*. Vet Med Int. 2022;2022:7626618.
24. Ayalew M, Bekele A. Evaluation of the antimalarial activity of crude extract of *Eucalyptus globulus* Labill. Leaf against *Plasmodium berghei* in mice. Evid Based Complement Alternat Med. 2021;2021:7068999.
25. Dibessa TT, Engidawork E, Nedi T, Teklehaymanot T. Antimalarial activity of the aqueous extract of the latex of *Aloe pirottae* Berger. (Aloaceae) against *Plasmodium berghei* in mice. J Ethnopharmacol. 2020;255:112763.
26. Krettli AU, Adebayo JO, Krettli LG. Testing of natural products and synthetic molecules aiming at new antimalarials. Curr Drug Targets. 2009;10:261-70.

27. Deharo E, Bourdy G, Quenevo C, Muñoz V, Ruiz G, Sauvain M. A search for natural bioactive compounds in Bolivia through a multidisciplinary approach. Part V. Evaluation of the antimalarial activity of plants used by the Tacana Indians. *J Ethnopharmacol.* 2001;77:91-8.
28. Gupta M, Mishra A. Bioactive flavonoids: A comparative overview of the biogenetic and chemical synthesis approach. *Mini Rev Med Chem.* 2023.
29. Omar F, Tareq AM, Alqahtani AM, Dhama K, Sayeed MA, Emran TB, et al. Plant-based indole alkaloids: A comprehensive overview from a pharmacological perspective. *Molecules.* 2021;26:2297.
30. Degotte G, Pirotte B, Francotte P, Frédérick M. Overview of natural Antiplasmodials from the last decade to inspire medicinal chemistry. *Curr Med Chem.* 2021;28:6199-233.
31. Weenen H, Nkunya MH, Bray DH, Mwasumbi LB, Kinabo LS, Kilimali VA, et al. Antimalarial compounds containing an alpha,beta-unsaturated carbonyl moiety from Tanzanian medicinal plants. *Planta Med.* 1990;56:371-3.
32. Kamala A, Middha SK, Karigar CS. Plants in traditional medicine with special reference to *Cyperus rotundus* L.: a review. *3 Biotech.* 2018;8:309.
33. Rehman K, Lötsch F, Kremsner PG, Ramharter M. Haemolysis associated with the treatment of malaria with artemisinin derivatives: a systematic review of current evidence. *Int J Infect Dis.* 2014;29:268-73.
34. Savargaonkar D, Das MK, Verma A, Mitra JK, Yadav CP, Srivastava B, et al. Delayed haemolysis after treatment with intravenous artesunate in patients with severe malaria in India. *Malar J.* 2020;19:39.
35. Salehi M, Masoumi-Asl H, Assarian M, Khoshnam-Rad N, Haghi AM, Nikbakht M, Khalili H. Delayed hemolytic anemia after treatment with artesunate: case report and literature review. *Curr Drug Saf.* 2019;14:60-6.
36. Sardar S, Abdurabu M, Abdelhadi A, Habib MB, Jamshaid MB, Hajjar AH, et al. Artesunate-induced hemolysis in severe complicated malaria - A diagnostic challenge: A case report and literature review of anemia in malaria. *IDCases.* 2021;25:e01234.
37. Ounjaijean S, Somsak V. Synergistic antimalarial treatment of *Plasmodium berghei* infection in mice with dihydroartemisinin and *Gymnema inodorum* leaf extract. *BMC Complement Med Ther.* 2023;23:20.
38. Ounjaijean S, Somsak V. Combination of zingerone and dihydroartemisinin presented synergistic antimalarial activity against *Plasmodium berghei* infection in BALB/c mice as in vivo model. *Parasitol Int.* 2020;76:102088.

Figures

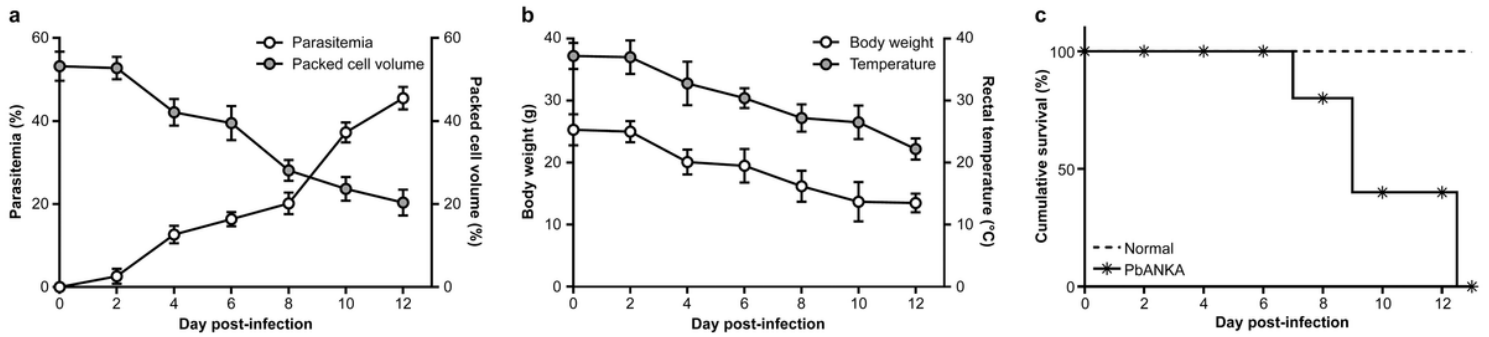


Figure 1

PbANKA infection in Balb/c mice. Mice were infected with PbANKA and parameters including (a) parasitemia and PCV (b) BW and rectal temperature (c) survival time were then investigated as previously described in Methods section. Results were expressed as mean $\pm$ SEM (n = 5).

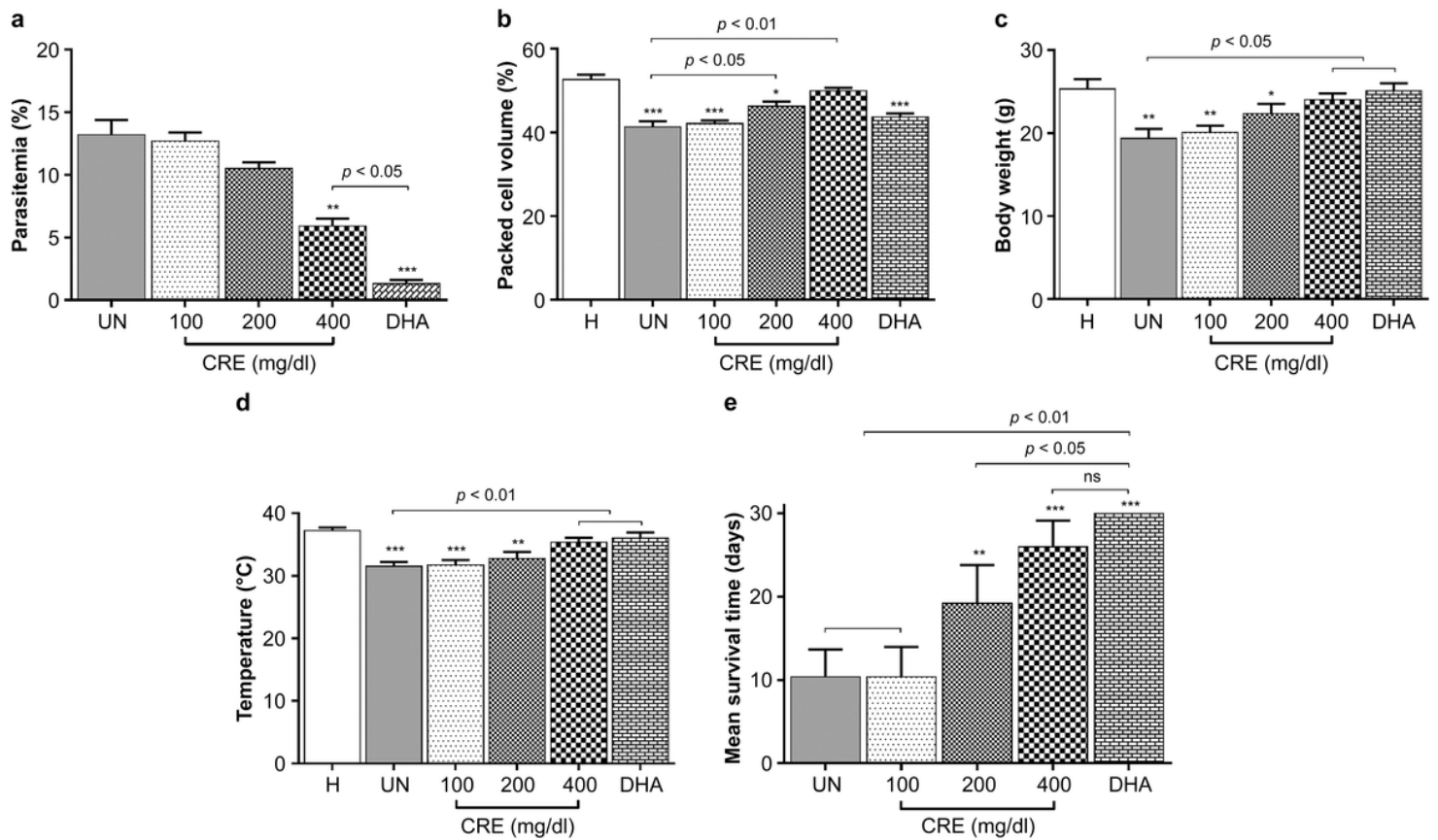


Figure 2

Four-day suppressive antimalarial activity of CRE against PbANKA infection in Balb/c mice. Mice were infected with PbANKA and administered orally with 100, 200, and 400 mg/kg of CRE for 4-consecutive days as previously described in Methods section. (a) Parasitemia (b) PCV (c) BW, and (d) rectal temperature were measured. Moreover, (e) MST was also investigated for 30 days. Results were expressed as mean $\pm$ SEM (n = 5). * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ compared to UN or H. UN;

untreated control, H; healthy control, 100, 200, and 400 CRE; aqueous crude extract of *C. rotundus* at doses of 100, 200, and 400 mg/kg, respectively, DHA; 5 mg/kg of dihydroartemisinin, and ns; not significant.

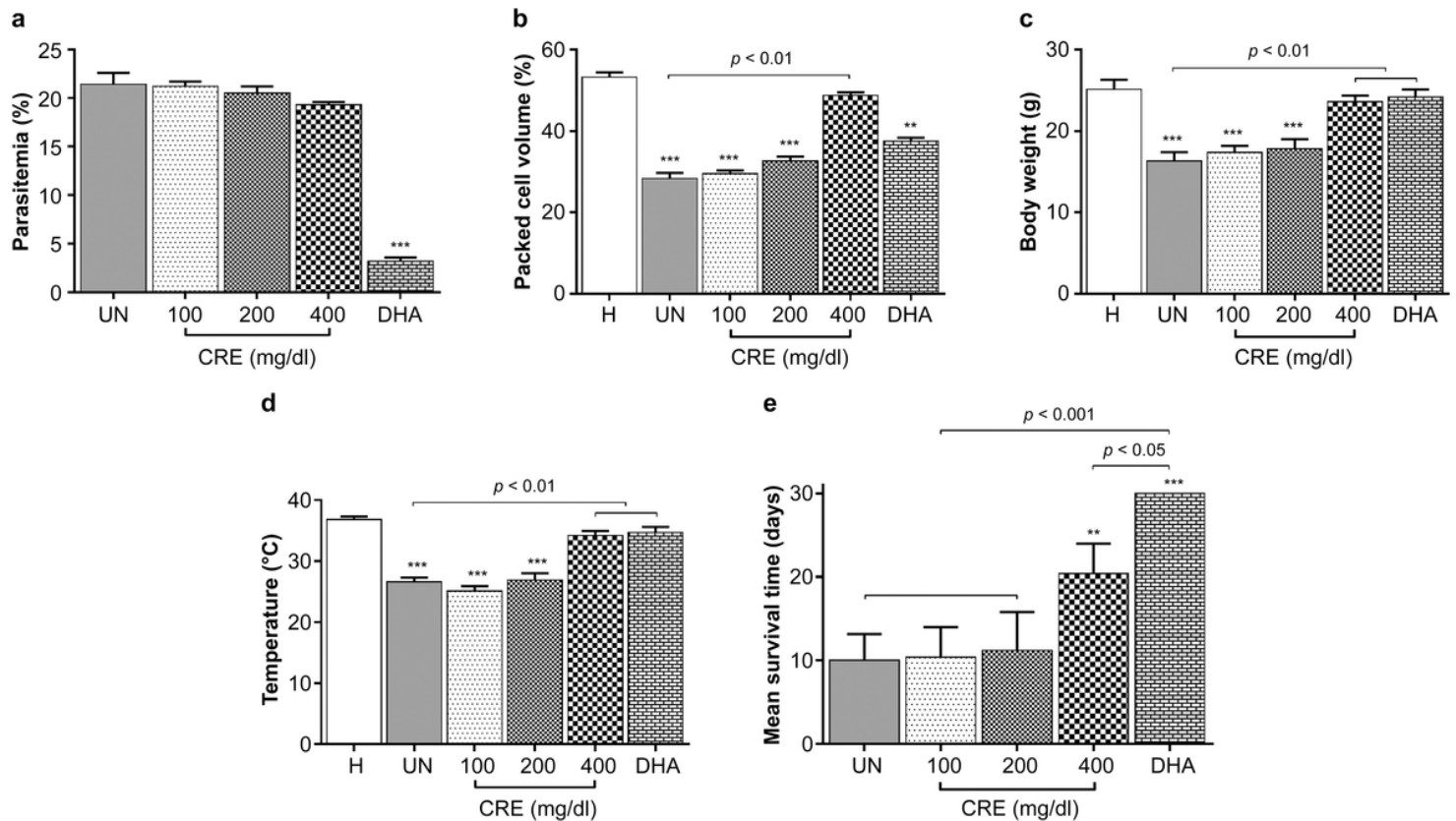


Figure 3

Curative antimalarial activity of CRE against PbANKA infection in Balb/c mice. Mice were inoculated with PbANKA for 4 days and administered orally with 100, 200, and 400 mg/kg of CRE for 4-consecutive days as previously described in Methods section. (a) Parasitemia (b) PCV (c) BW, and (d) rectal temperature were measured. Moreover, (e) MST was also investigated for 30 days. Results were expressed as mean $\pm$ SEM (n = 5). ** $p < 0.01$, and *** $p < 0.001$ compared to UN or H. UN; untreated control, H; healthy control, 100, 200, and 400 CRE; aqueous crude extract of *C. rotundus* at the doses of 100, 200, and 400 mg/kg, respectively, and DHA; 5 mg/kg of dihydroartemisinin.

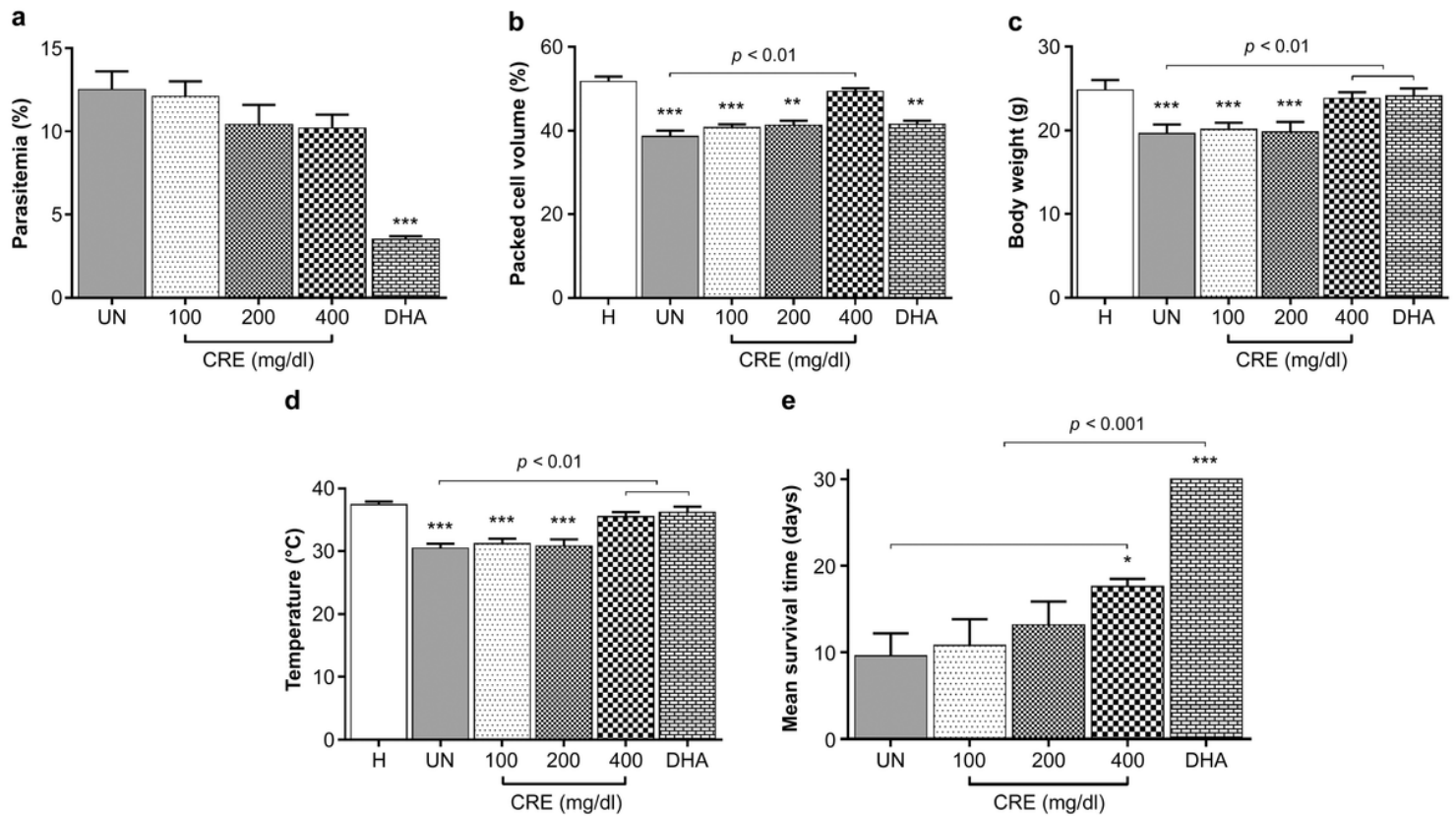


Figure 4

Prophylactic antimalarial activity of CRE against PbANKA infection in Balb/c mice. Mice were administered orally with 100, 200, and 400 mg/kg of CRE for 4-consecutive days and then infected with PbANKA for 4 days as previously described in Methods section. (a) Parasitemia (b) PCV (c) BW, and (d) rectal temperature were measured. Moreover, (e) MST was also investigated for 30 days. Results were expressed as mean $\pm$ SEM ($n = 5$). ** $p < 0.01$, and *** $p < 0.001$ compared to UN or H. UN; untreated control, H; healthy control, 100, 200, and 400 CRE; aqueous crude extract of *C. rotundus* at the doses of 100, 200, and 400 mg/kg, respectively, and DHA; 5 mg/kg of dihydroartemisinin.

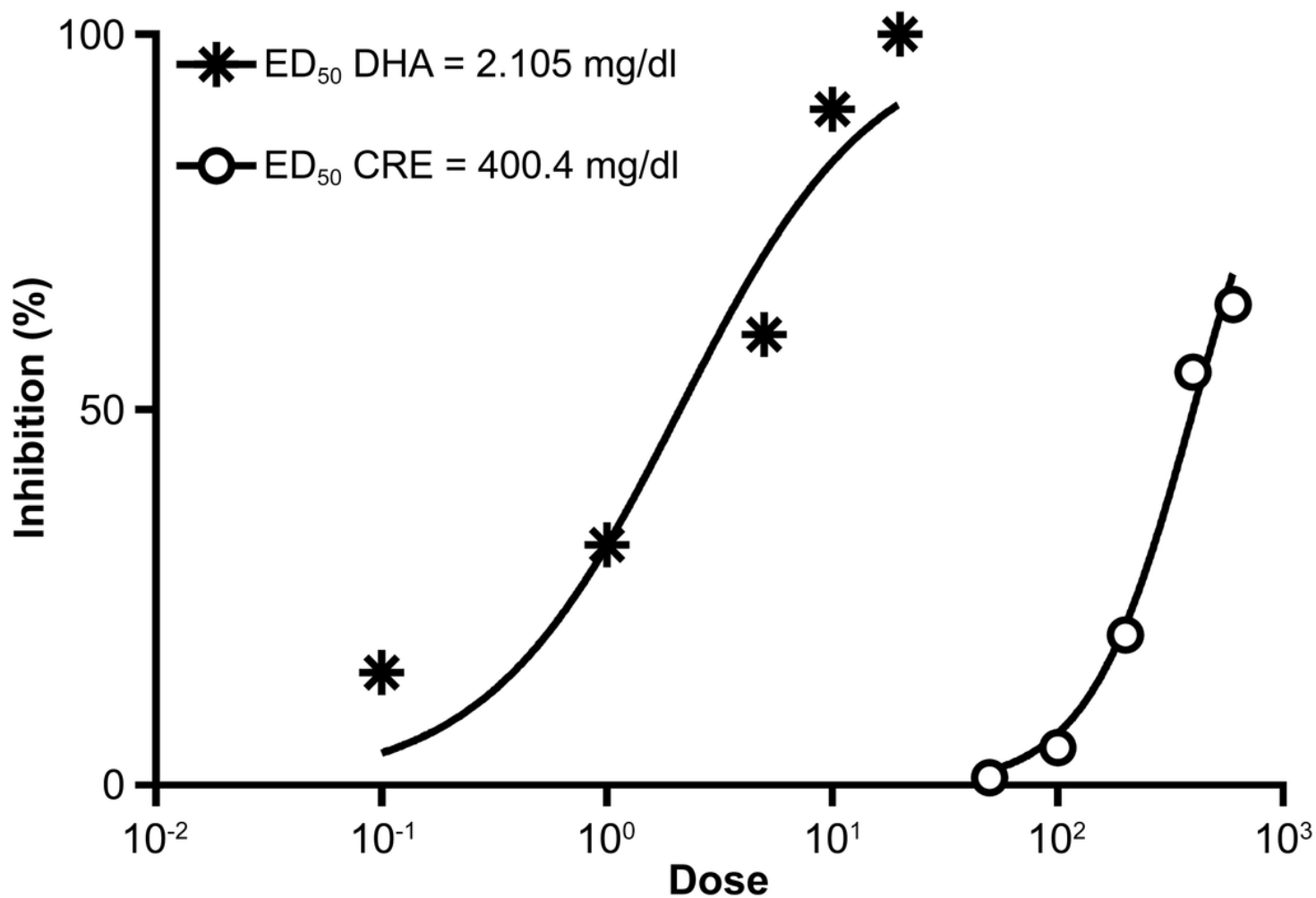


Figure 5

Effective dose of DHA and CRE against PbANKA infection in Balb/c mice. For investigation of ED_{50} value, 4-day suppressive test was carried out as previously described in Methods section. Results were expressed as mean $\pm$ SEM ($n = 5$). DHA, dihydroartemisinin; CRE, aqueous crude extract of *C. rotundus*.

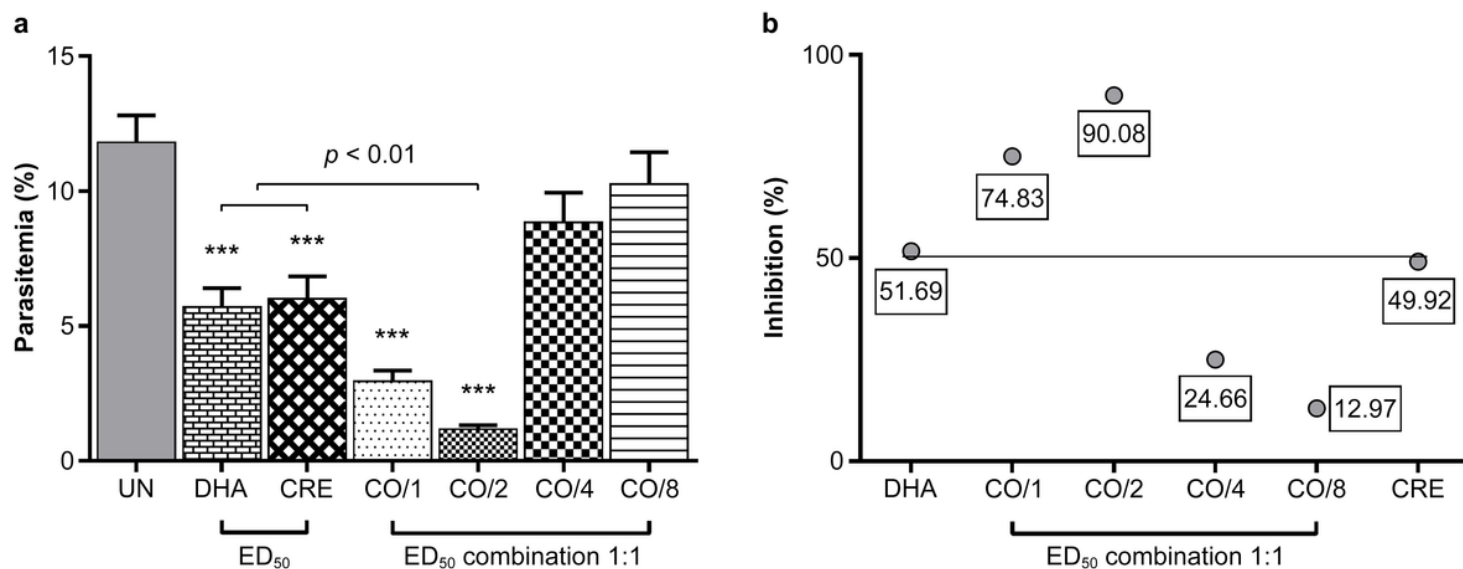


Figure 6

Combination antimalarial activity of DHA and CRE against PbANKA infection in Balb/c mice. Mice were infected with PbANKA and given orally with DHA and CRE combination (1:1) at ED_{50} , $ED_{50/2}$, $ED_{50/4}$, and $ED_{50/8}$ values for 4-consecutive days. (A) parasitemia was measured and (B) interaction line was generated. Results were expressed as mean $\pm$ SEM ($n = 5$). *** $p < 0.001$ compared to UN. UN; untreated control, DHA; dihydroartemisinin, CRE; aqueous crude extract of *C. rotundus*, CO/1, CO/2, CO/4, and CO/8; combination treatment of DHA and CRE at ED_{50} , $ED_{50/2}$, $ED_{50/4}$, and $ED_{50/8}$ values, respectively.