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Research Article

In Vivo Antimalarial Activity of *Cyperus rotundus* and Its Combination with Dihydroartemisinin against *Plasmodium berghei*

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Background. The increase in the number of drug-resistant *Plasmodium* species continues to be a serious public health concern. Therefore, identification of the 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 using 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 ED50/2 (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. Introduction

Malaria remains one of the most serious diseases and is a significant public health concern. Malaria is a disease that is attributed to the transmission of a *Plasmodium* parasite through female *Anopheles* mosquitoes. In humans, there are five specific *Plasmodium* species that have the ability to infect individuals, namely, *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 must be 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, anti-hyperglycemic, antihyperlipidemic, antiulcer, antimicrobial, antihelmintic, 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 has not yet been investigated in animal models. Therefore, the aim of this study was to evaluate the effectiveness of the aqueous crude extract of *C. rotundus*, both alone and in combination with dihydroartemisinin, in treating mice that were infected with *P. berghei*.

2. Materials and 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 (NRU65/09-010) 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. The dried material was then reduced to coarse powder and subsequently prepared as a fine powder using an electric blender (Mettler-Toledo, AB204-S, SN 1120510763). 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 (SUSPA lifeline, AFB1224SHE, SN MU-E70-91031) and filtered through a Whatman no.1 filter paper. The filtrate was then centrifuged (Hettich Zentrifugen, ROTANTA 460RF, SN 0000813-06) at 4,000 *g* for 10 min, and supernatant was collected. The supernatant was lyophilized (Martin Chist, Alpha 1-4 LSCpkus, SN 20464) to obtain an 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). The 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 (paw licking, salivation, stretching of the entire body, weakness, respiratory distress, and coma) or mortality.

2.5. Subacute Toxicity Testing. The procedures described in Guide 407 of the OECD guidelines were performed using the limit test with 1,000 mg/kg of CRE [13]. BALB/c mice ($n = 5$) were administered a daily dose of 1,000 mg/kg of CRE 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 a 10% Giemsa dye solution. Parasitized erythrocytes were identified under a light microscope with a 100× oil immersion lens. Parasitemia was calculated using the following equation:

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

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 were recorded, up to a maximum of 30 days. The mean survival time (MST) was calculated using the following equation:

$$\text{MST} = \frac{\text{Sum of survival time of all mice in a group}}{\text{Total number of mice in that group}}. \quad (2)$$

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 the following equation:

$$\%\ \text{PCV} = \frac{\text{Volume of total erythrocytes in a given volume of blood}}{\text{Total blood volume}} \times 100. \quad (3)$$

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 of CRE on early infection of PbANKA in mice was evaluated by Peter's 4-day suppressive test [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 the 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 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 the following:

$$\%\ \text{inhibition} = \frac{(\text{parasitemia of the untreated group} - \text{parasitemia of the tested group})}{\text{parasitemia of the untreated group}} \times 100. \quad (4)$$

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 experimental groups were treated with CRE (100, 200, and 400 mg/kg) orally by gavage. On the other hand, the untreated and positive controls 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 then calculated. Moreover, the optimal ED_{50} value was determined by using a nonlinear regression for estimating the dose-response variable slope.

2.16. Combination Antimalarial Treatment. The ED_{50} values of CRE and DHA were used to evaluate the combination treatment. The CRE and DHA were combined at a 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 was calculated. Points above the joint line indicated synergism while those around the line or below indicated additive or antagonistic interactions, respectively. A combination index (CI) value was also performed.

2.17. Statistics. Results are displayed as the mean ± standard error of the mean (SEM). Using nonlinear regression for the dose-response variable slope, the optimal ED_{50} value was determined. A one-way ANOVA with Tukey's post hoc test was used to compare the 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 of 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 injuries (Table 1). Consequently, the LD_{50} value for CRE was >2,000 mg/kg, and CRE was subsequently considered safe for oral administration.

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% (Figure 1(a)). In addition, BW and rectal temperature were considerably decreased in PbANKA-infected mice (Figure 1(b)), and all infected mice died within two weeks (Figure 1(c)). 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% (Figure 2(a)). DHA-treated mice exhibited a significant ($p < 0.05$) decrease in PCV (43.7 ± 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 ± 0.7%) compared with that in the healthy control (52.6 ± 1.2%) (Figure 2(b)). 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 ± 0.9°C) when compared with these parameters in the healthy control (25.3 ± 1.2 g and 37.2 ± 0.5°C, respectively) (Figures 2(c) and 2(d)). 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) (Figure 2(e)). However, mice treated with 100 and 200 mg/kg of CRE exhibited significant ($p < 0.05$) decreases in PCV, BW, and rectal temperature (Figures 2(b)–2(e)). With prolonged MST, 400 mg/kg of CRE exerted chemosuppressive antimalarial activity and protective effects on PCV reduction, BW loss, and rectal temperature decrease.

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 the mean ± SEM ($n = 5$).

3.4. Curative Antimalarial Effect of CRE in PbANKA-Infected Mice. The parasitemia level did not significantly decrease at any of the three CRE doses utilized to treat mice (Figure 3(a)). In comparison to the healthy control group, mice that were administered with 400 mg/kg of CRE did not exhibit statistically significant decreases in PCV (48.8 ± 0.7%), BW (23.6 ± 0.8 g), or rectal temperature (34.2 ± 0.6°C). However, a significant reduction ($p < 0.01$) in PCV (37.5 ± 0.9%) was observed in the group treated with DHA (Figures 3(b)–3(d)). Moreover, the MST of mice that received a dosage of 400 mg/kg of CRE was found to be greater (20.4 ± 3.6 days) compared to the untreated mice (10.0 ± 3.2 days) (Figure 3(e)).

3.5. Prophylactic Antimalarial Effect of CRE on PbANKA-Infected Mice. CRE lacked chemoprophylactic activity at all three doses in PbANKA-infected mice (Figure 4(a)). Compared with that in the untreated control, only the 400 mg/kg CRE-treated group demonstrated a protective effect on PCV (49.4 ± 0.7%), BW (23.8 ± 0.6 g), and rectal temperature reduction (35.5 ± 0.4°C) (Figures 4(b)–4(d)). PCV remained significantly ($p < 0.01$) reduced in the DHA-treated group (41.5 ± 0.9%) (Figure 4(b)). 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) (Figure 4(e)). 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 (Figure 5). The combination of DHA and CRE at ED_{50} and $ED_{50/2}$ exhibited significant ($p < 0.001$) antimalarial activity compared with that of the untreated control with 74.83% and 90.08% inhibition of parasites, respectively (Figure 6(a)). 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,

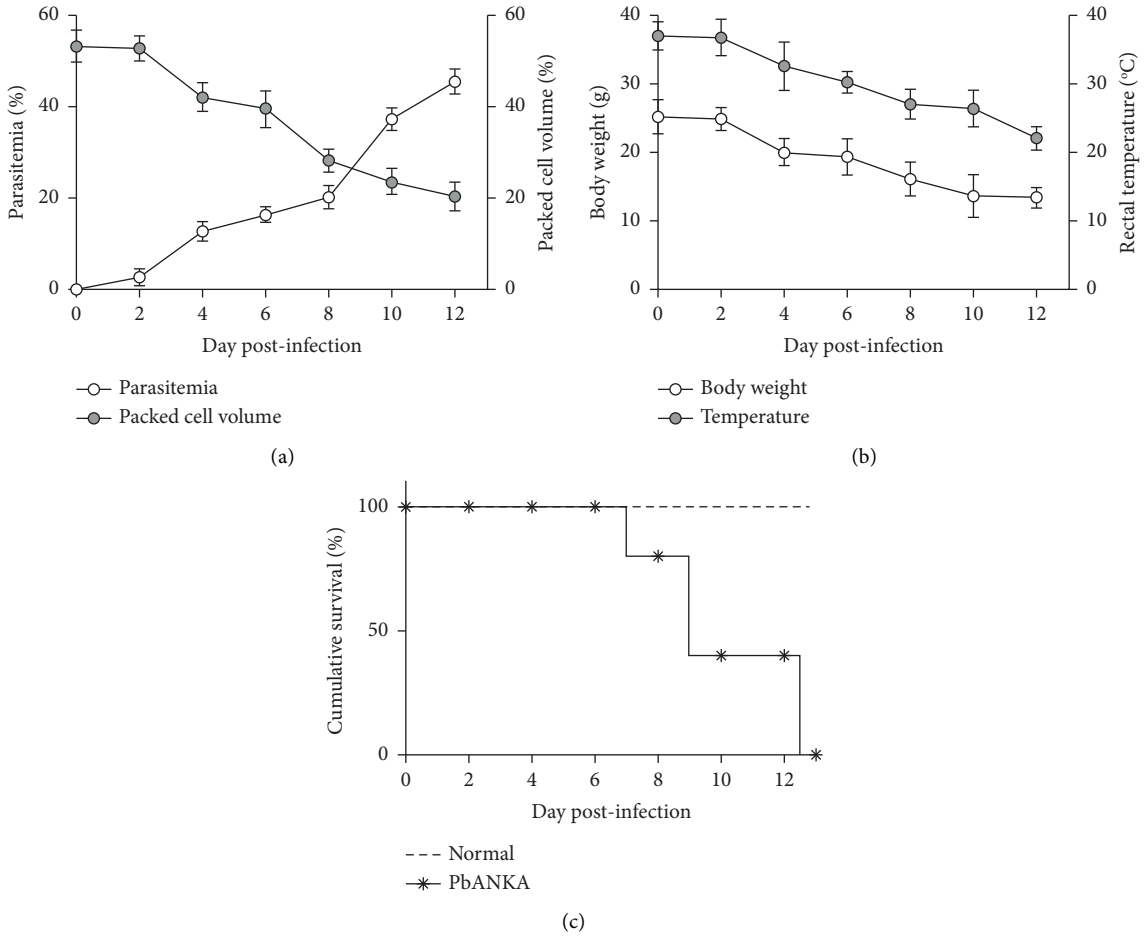


Figure 1: PbANKA infection in Balb/c mice. Mice were infected with PbANKA and parameters including (a) parasitemia and PCV, (b) BW and rectal temperature, and (c) survival time were then investigated as previously described in Methods section. Results were expressed as the mean ± SEM ($n = 5$).

synergistic interaction of the combination treatment at the ED_{50} and $ED_{50/2}$ doses (CI = 0.92488 and 0.21701, respectively) was observed as indicated by the CI value of <1.0 (Table 2) and the interaction points above the joint line (Figure 6(b)). Nonetheless, the $ED_{50/4}$ and $ED_{50/8}$ (CI = 1.85871 and 2.34981, respectively) of DHA and CRE combination demonstrated antagonistic interaction.

4. Discussion

Recent resistance to artemisinin and its derivatives has emerged in Southeast Asia. Consequently, new antimalarial drug candidates, 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 LD_{50} >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 LD_{50} 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]. In addition, 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

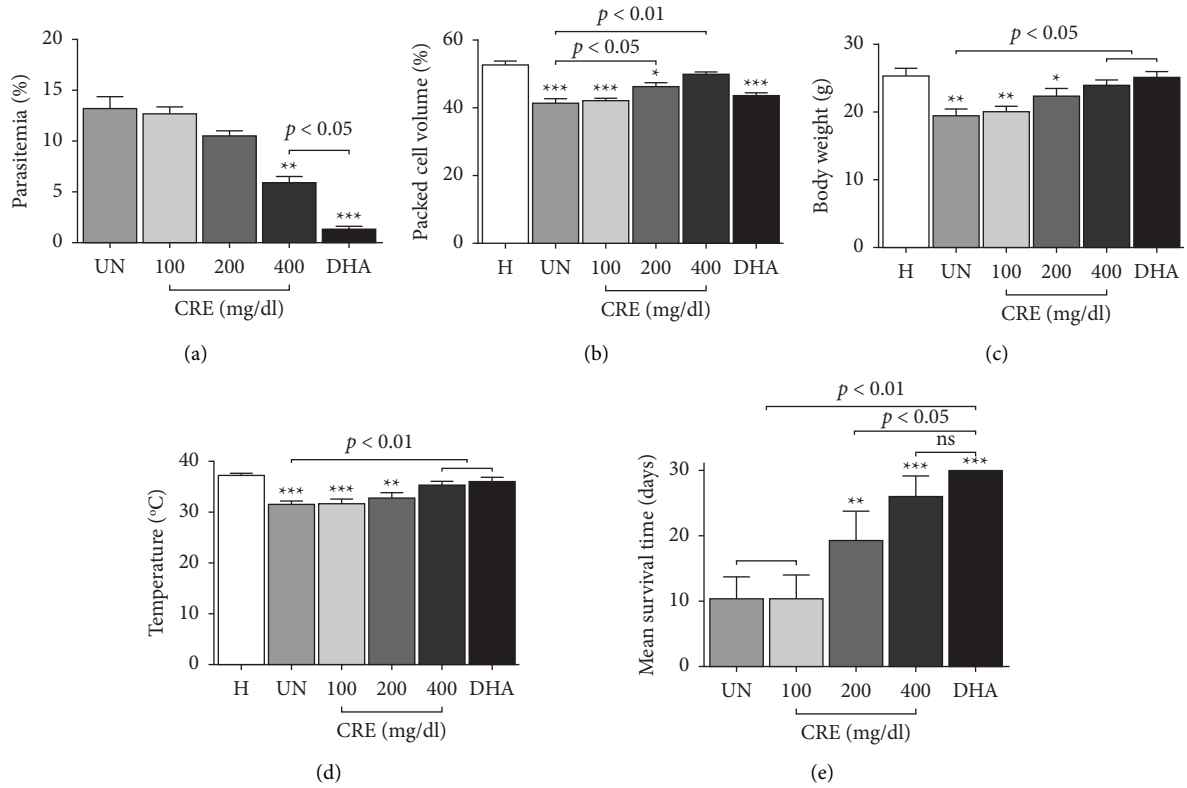


Figure 2: Four-day suppressive antimalarial activity of CRE against PbANKA infection in Balb/c mice. Mice were infected with PbANKA and administered orally with 100, 200, and 400 mg/kg of CRE for 4-consecutive days as previously described in Methods section. (a) Parasitemia, (b) PCV, (c) BW, and (d) rectal temperature were measured. Moreover, (e) MST was also investigated for 30 days. Results were expressed as the mean ± 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; ns: not significant.

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 and 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 mitigates the early infection by PbANKA. General extracts that inhibit parasitemia by ≥30% are considered active [26]. *In vivo* antimalarial activity can be classified as moderate, good, or very good if the percentage of inhibition is ≥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 patchoulenone, 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

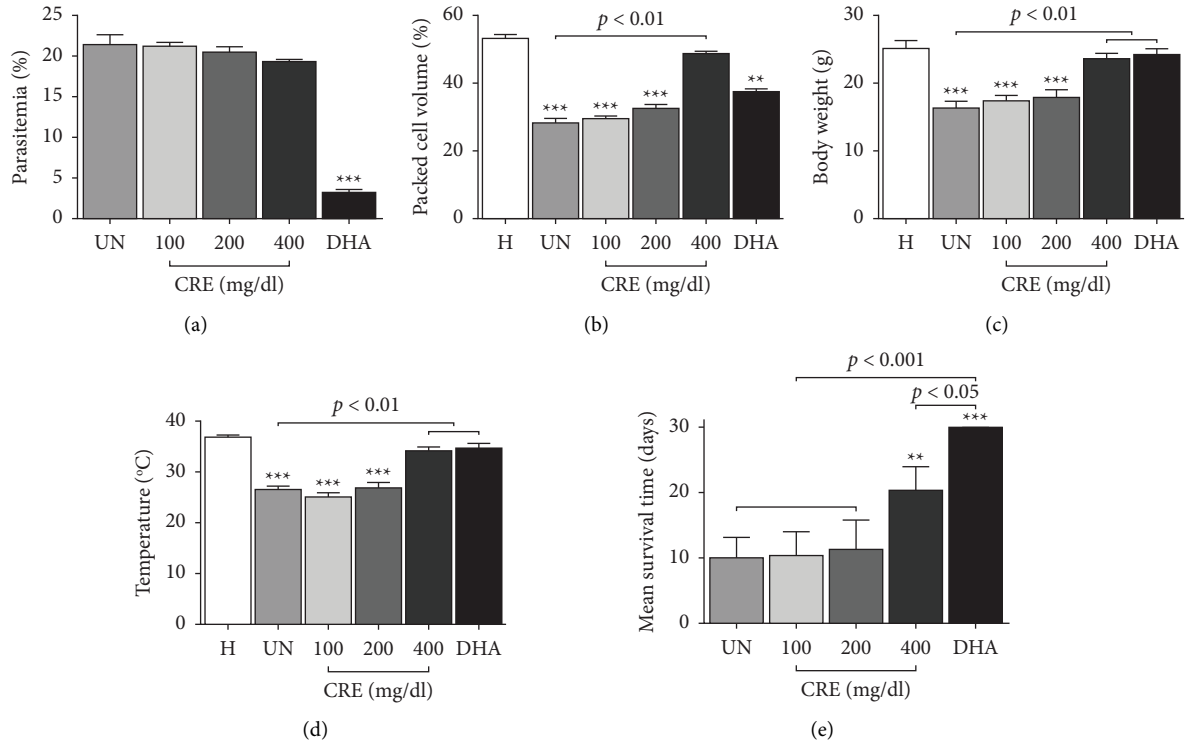


Figure 3: Curative antimalarial activity of CRE against PbANKA infection in Balb/c mice. Mice were inoculated with PbANKA for 4 days and administered orally with 100, 200, and 400 mg/kg of CRE for 4-consecutive days as previously described in Methods section. (a) Parasitemia, (b) PCV (c) BW, and (d) rectal temperature were measured. Moreover, (e) MST was also investigated for 30 days. Results were expressed as the mean ± 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; DHA: 5 mg/kg of dihydroartemisinin.

erythrocyte invasion by parasites, inhibition of parasite growth and multiplication, blocked entry of nutrients into 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 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 reduction of its concentration in the body, and it could also be related to the rapid multiplication of the parasite in an established infection, where the parasite is growing exponentially. In addition, 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]. In addition, artemisinin-induced and postartemisinin-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 by which 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

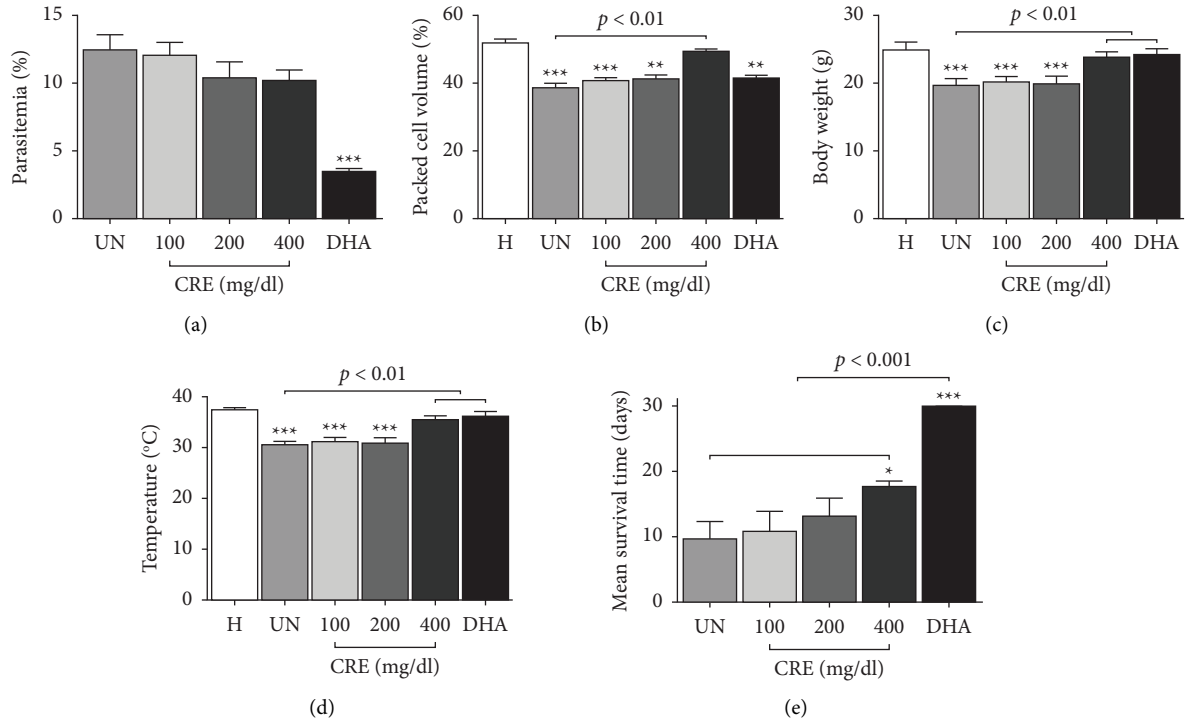


Figure 4: Prophylactic antimalarial activity of CRE against PbANKA infection in Balb/c mice. Mice were administered orally with 100, 200, and 400 mg/kg of CRE for 4-consecutive days and then infected with PbANKA for 4 days as previously described in Methods section. (a) Parasitemia (b) PCV (c) BW, and (d) rectal temperature were measured. Moreover, (e) MST was also investigated for 30 days. Results were expressed as the mean ± 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; DHA: 5 mg/kg of dihydroartemisinin.

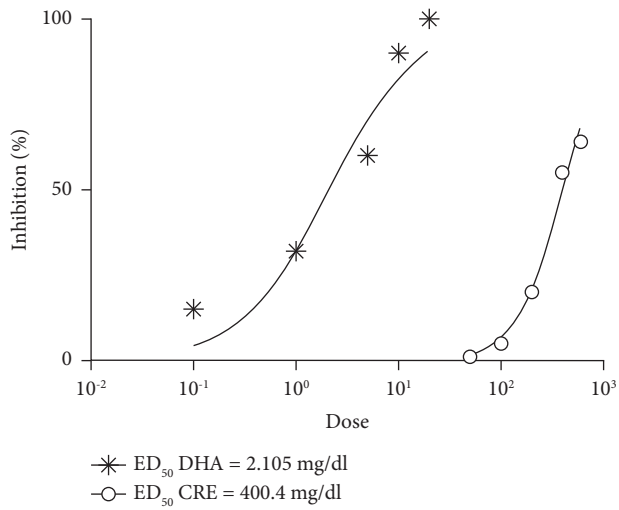


Figure 5: Effective dose of DHA and CRE against PbANKA infection in Balb/c mice. For investigation of ED_{50} value, the 4-day suppressive test was carried out as previously described in Methods section. Results were expressed as the mean ± SEM ($n = 5$). DHA, dihydroartemisinin; CRE, aqueous crude extract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

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, the activity of 100 and 200 mg/kg of CRE was insufficient to prevent these abnormalities in PbANKA-infected mice.

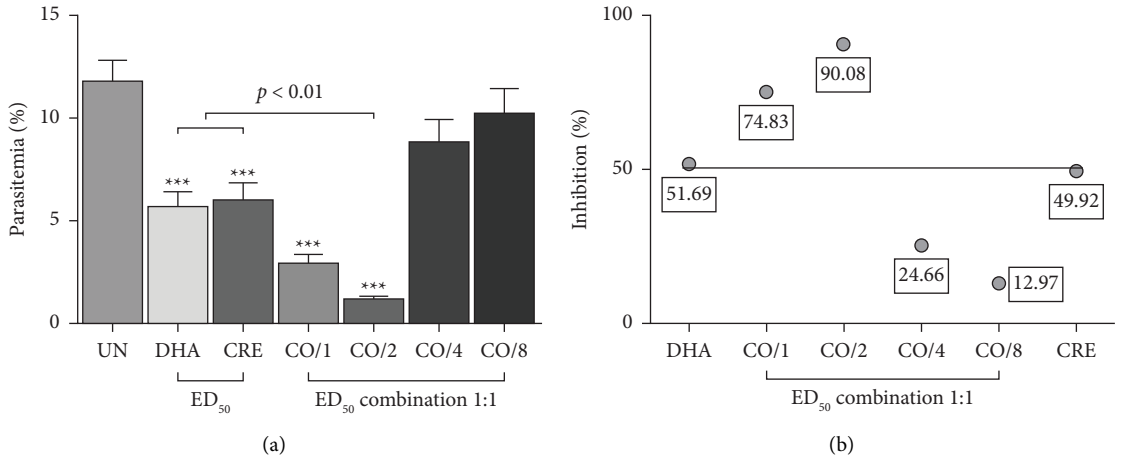


Figure 6: Combination antimalarial activity of DHA and CRE against PbANKA infection in Balb/c mice. Mice were infected with PbANKA and given orally with DHA and CRE combination (1 : 1) at ED_{50}, $ED_{50/2}$, $ED_{50/4}$, and $ED_{50/8}$ values for 4 consecutive days. (a) Parasitemia was measured and (b) interaction line was generated. Results were expressed as the mean ± 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.

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.

MST is an 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 a 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 doses of $ED_{50/2}$ showed more successful chemotherapeutic treatment in the PbANKA-infected mouse model. In addition, 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 variant
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.

Data Availability

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.

Ethical Approval

All experimental protocols involving animal were reviewed and approved by the 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).

Disclosure

A preprint has previously been published [39].

Conflicts of Interest

The authors declare that there are no conflicts of interest.

Authors' Contributions

Sakaewan Ounjaijean proposed the methodology, conducted formal analysis, performed investigation, provided the resources, visualized the study, curated the data, and wrote, reviewed, and edited the manuscript. Charupa Lektip curated the data and wrote, reviewed, and edited the manuscript. Voravuth Somsak conceptualized the study, proposed the methodology, validated the study, wrote the original draft, and supervised the study All authors read and approved the final manuscript.

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