TROPICAL LIFE SCIENCES RESEARCH

Caffeine Prolongs Survivability And Significantly Modulates Cytokine Levels of TNF-α, IFN-γ, IL-18 and IL-10 in *Plasmodium berghei* ANKA-infected ICR Mice

Authors:

Ahmad Zakwan Noriman, Mohd Ridzuan Mohd Abd Razak, Wan Nurhayati Wan Hanafi, Rusliza Basir, Hani Kartini Agustar and Wan Rozianoor Mohd Hassan*

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Highlights

- The lowest concentration of caffeine (5 mg/kg b.w.) showed the most effective chemosuppression at 64.18 ± 9.17% as compared to other concentrations (10 and 20 mg/kg b.w.) in *P. berghei* ANKA-infected ICR mice.

- Caffeine at 5 mg/kg b.w. prolongs survivability (17 days) of *P. berghei* ANKA-infected ICR mice.

- Caffeine significantly modulates levels of pro- and anti-inflammatory cytokines (TNF-α, IFN-γ, IL-18 and IL-10) in cerebral malaria murine model.

EARLY VIEW

Caffeine Prolongs Survivability and Significantly Modulates Cytokine Levels of TNF-α, IFN-γ, IL-18 and IL-10 in *Plasmodium berghei* ANKA-infected ICR Mice

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Running head: Caffeine effects in *P. berghei* ANKA-infected ICR mice

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Abstract: Caffeine, a bioactive compound in *Theobroma cacao, exhibits* various pharmacological activities, including anti-inflammatory property. Malaria is an inflammatory-related disease caused by *Plasmodium spp*. Severe infection of this disease can cause cerebral malaria, which is characterized by neuroinflammation. An *in vitro* study of caffeine suggested that it possesses anti-malarial property. However, caffeine's potential in both anti-malarial and cytokine-modulating properties through *in vivo* have yet to be explored. This study investigated anti-malarial and cytokine-modulating effects of caffeine in *P. berghei*

ANKA-infected ICR mice. Anti-malarial activity of caffeine at 5, 10, and 20 mg/kg body weight were given via intraperitoneal (i.p.) injection and the chemosuppressive percentage (percentage of parasitemia inhibition) was calculated based on Peter and Robinsons (1992). In addition, the cytokine-modulating effect was determined using the ELISA method. Chemosuppressive test at 5, 10 and 20 mg/kg b.w. of caffeine in the *P. berghei* ANKA-infected ICR mice exhibited 64.18 ± 9.17, 39.93 ± 5.63, and 21.86 ± 5.57%, respectively. Notably, the lowest concentration, 5 mg/kg b.w. of caffeine was able to prolong significantly ($p < 0.05$) the survivability of malaria-infected ICR mice for 17 days as compared to infected mice (12 days). This effective dose of caffeine revealed a significant reduction in cytokine levels of TNF- α, IFN- γ, IL-18, and IL-10 by 1.08-, 1.12-, 1.12-, and 1.15-folds as compared to malaria-infected mice. Collectively, 5 mg/kg b.w. of caffeine exhibited good chemosuppressive, modulated levels of inflammatory cytokines and prolonged survivability of *P. berghei* ANKA-infected ICR mice. Hence, caffeine can be a plausible candidate for the development of anti-malarial therapeutic drugs.

Keywords: Caffeine, Cytokine-Modulation, ICR mice, Malaria, *P. berghei* ANKA

Abstrak: Kafein, sebatian bioaktif dalam *Theobroma cacao*, mempamerkan pelbagai aktiviti farmakologi, termasuk sifat anti-radang. Malaria merupakan penyakit berkaitan keradangan yang disebabkan oleh *Plasmodium spp*. Jangkitan ketara penyakit ini boleh menyebabkan malaria serebrum, yang dicirikan oleh keradangan saraf. Kajian *in vitro* kafein mencadangkan bahawa ia mempunyai kandungan anti-malaria. Walau bagaimanapun, potensi kafein yang mempunyai aktiviti anti-malaria dan modulasi sitokin dalam *in vivo* masih belum diterokai. Kajian ini bertujuan untuk mengenal pasti kesan anti-malaria dan modulasi sitokin kafein dalam mencit ICR yang dijangkiti *P. berghei* ANKA. Aktiviti anti-malaria kafein pada 5, 10, dan 20 mg/kg berat badan (b.b.) diberikan melalui suntikan intraperitoneum (i.p.) dan peratusan kemosupresif (peratusan perencatan parasitemia) dikira berdasarkan Peter dan Robinsons (1992). Tambahan pula, kesan modulasi sitokin ditentukan menggunakan kaedah ELISA. Ujian kemosupresif pada 5, 10 dan 20 mg/kg b.b. kafein dalam mencit ICR yang dijangkiti *P. berghei* ANKA menunjukkan 64.18 ± 9.17, 39.93 ± 5.63, dan 21.86 ± 5.57% masing-masing. Khususnya, kafein dengan kepekatan terendah, 5 mg/kg b.b. dapat memanjangkan dengan ketara ($p < 0.05$) kemandirian mencit ICR yang dijangkiti malaria selama 17 hari berbanding dengan mencit yang dijangkiti (12 hari). Rawatan dengan dos kafein yang berkesan ini mendedahkan pengurangan ketara dalam aras sitokin TNF- α, IFN- γ, IL-18, dan IL-10 berbanding mencit yang dijangkiti malaria. Secara kolektif, kafein pada 5 mg/kg b.b. mempamerkan penindasan kemosupresif yang baik, memodulasi aras keradangan sitokin

dan memanjangkan kemandirian mencit ICR yang dijangkiti *P. berghei* ANKA. Oleh itu, kafein boleh menjadi calon pembangunan ubat terapeutik anti-malaria.

Kata kunci: Kafein, Modulasi Sitokin, Mencit ICR, Malaria, *P. berghei* ANKA

INTRODUCTION

Malaria is an infectious disease globally, as 95% of the diseases accounted for are from major regions of African countries (World Health Organization, 2024). In 2023, 263 million infections and 597,000 deaths worldwide were reported due to this inflammatory-related disease (World Health Organization, 2024). *P. knowlesi, P. ovale, P. falciparum, P. vivax,* and *P. malariae* are parasite species that can infect humans (Lee *et al.*, 2022). A severe complication of malaria infection is cerebral malaria, which is characterized by the sequestration of parasitized red blood cells (pRBC) within the microcirculation and elevated pro-inflammatory cytokines such as IFN-γ, TNF-α, and IL-18 (Ali *et al*., 2022; Basir *et al*., 2012).

Chloroquine (CQ), a common malaria treatment, is synthesized from quinoline that was found in Cinchona bark in the early 19th century (Zhou *et al*., 2020). The emergence and spread of drug-resistant *Plasmodium* parasites, such as CQ and artemisinin, can also contribute to the increase in malaria cases (Parija and Antony, 2016). Rising cases of anti-malarial drug resistance prompted the need to develop and improve drugs for treatment, especially from medicinal plants (Rasmussen *et al.,* 2022; Titanji *et al*., 2008; Vaou *et al*., 2021). Many anti-malarial drug development studies have been conducted from plant resources (Habibi *et al*., 2022). Latest research on malaria includes exploration of natural plant such as methanol extract of *Picralima nitida* in Swiss albino mice infected with *P. berghei* and ethanol extract of *Blighia sapida* K.D. Koenig in Swiss albino mice infected with CQ-resistant *P. berghei* ANKA MRA 311 (Ogbeiden *et al.,* 2023; Akinnyemi *et al.,* 2025).

1,3,7-trimethylxanthine, or caffeine, is a major bioactive compound that is consumed daily by humans in the form of coffee, tea, or chocolate (Dorvigny *et al*., 2022; Temple *et al*., 2017). Caffeine has been known as a stimulant in the central nervous system (Chen *et al*., 2010). Besides that, caffeine has been identified as having protective effects against neurodegenerative diseases by preventing and stabilizing the blood-brain barrier to ensure that the barrier remains intact (Chen *et al*., 2010). The alkaloid also has shown anti-inflammatory and neuroprotective abilities (Barcelos *et al*., 2020; Kolahdouzan and Hamadeh, 2017). In addition, caffeine is an A2AR antagonist which competes with adenosine to bind to the receptor. Caffeine binding to A2AR receptor inhibited the binding of adenosine which caused A2AR not to be activated. This further blocked NF-Kβ which reduces inflammatory cytokines production and prevents neuroinflammation (Sonsalla *et al*., 2012).

Caffeine at 20 mg/mL pyrazolines has been identified to have potential for anti-malarial properties based on an *in vitro* study with a high inhibition percentage (85.2 ± 5.4%) against *P. falciparum* (Insuasty *et al*., 2015). However, caffeine's potential as an anti-malarial property and cytokine-modulating effect through *in vivo* model have yet to be explored. A previous study by Basir *et al.* (2012) was able to mimic the *P. falciparum* infection in humans using *Plasmodium berghei* ANKA in ICR mice (Basir *et al*., 2012). *P. berghei* ANKA is a widely used rodent model to study cerebral malaria, particularly in the context of evaluating anti-malarial properties and understanding immune responses (Craig *et al*., 2012). Thus, this study aims to investigate the anti-malarial and cytokine-modulating effects of caffeine in *P. berghei* ANKA-infected ICR mice.

MATERIALS AND METHODS

Parasite

P. berghei ANKA strain (CQ-sensitive) was procured from The Bioassay Unit, National Institute of Health (NIH), Shah Alam, Malaysia. A combination of cryogenic storage and passage in ICR mice was done to conserve the parasite at Universiti Teknologi MARA (UiTM) Shah Alam, Malaysia.

Treatment

Caffeine (1,3,7-trimethylxanthine; CAS No. 58-08-2) with a purity of 99.99% was purchased from Sigma-Aldrich and diluted in distilled water to prepare treatment concentrations of 5, 10, and 20 mg/kg b.w. The treatments were administered via i.p. injection. For the positive control, CQ (CAS No. 50-63-5), also obtained from Sigma-Aldrich at a purity of 99.99%, was diluted in distilled water and administered at 10 mg/kg b.w.

Experimental Mice

Male ICR mice weighing between 20-25 g were procured from Laboratory Animal Facility and Management (LAFAM), UiTM Puncak Alam, and were accommodated at Animal Lab House, UiTM Shah Alam. International guidelines for the use and maintenance of experimental animals were used for the maintenance and care of the mice. The rules and regulations set by the UiTM Committee on Animal Research and Ethics (UiTM CARE) of UiTM Shah Alam were followed for all procedures conducted on the animals (Ethical approval number: UiTM CARE: 399/2023 (3rd February 2023)).

Sample Size Calculation

In the experiment, a 10% dropout rate was considered to account for potential i.p. injection failures, as reported by Das and North (2007). The likelihood of unsuccessful parasite infection was not included in the calculation, as the probability of mice failing to become infected is less than 1% (Leitner et al., 2010). According to Basir et al. (2012), mortality in untreated infected mice increases only after the treatment period, with rates of 20% on day 5, 70% on day 6, and 100% by day 7. During the treatment procedure, the survival rate is typically 100%, as deaths occur after treatment is completed. Nonetheless, to accommodate the small possibility that a mouse might die during the 4-day treatment phase, a precautionary 5% mortality rate was included in the calculation.

a) Survivability test (Ali et al., 2017; Ali et al., 2022; Hassan et. al. 2019).

A total of six groups were used in the study, each consisting of seven mice (n=7), including a normal control, an infected control, a CQ-treated group (10 mg/kg), and three caffeine-treated groups (5, 10, and 20 mg/kg), requiring 42 male ICR mice. An additional four mice were needed for parasite culture. Allowances were also made for potential losses, including a 5% mortality rate among infected mice (2 mice) and a 10% i.p. injection failure rate (5 mice). In total, 53 male ICR mice aged 6–8 weeks were required for the study.

b) Cytokine Modulation activity (Ali et al., 2017; Ali et al., 2022; Hassan et. al. 2019).

In this study, a minimum of 40 male mice was required, consisting of four groups with 10 mice each: a normal control group, an infected group, an infected group treated with CQ, and an infected group treated with the effective dose of caffeine. An additional three mice were used for parasite culture prior to group inoculation. To account for potential losses, a 5% mortality rate was considered for the 30 infected mice, adding approximately two mice, and a 10% i.p. injection failure rate was applied to the 40 mice receiving injections, adding another four mice. Therefore, a total of 49 male mice were required for the study.

Anti-malarial Assay

Inoculum preparation

The serial passage of *P. berghei* was initiated based on a previous study (Huang *et al*., 2015). Stored blood parasites in Alservers' buffer solution were i.p. injected into normal mice at 0.2 mL and allowed for three to five days of inoculation. After being anaesthetized with carbon dioxide, a cardiac puncture was done to draw blood from infected mice. A volume of 0.2 mL containing $2x10^7$ pRBC was injected into a normal mouse as a subsequent passage. Blood was diluted with saline 0.85% to give $2x10^7$ pRBC in 0.2 mL of injection volume, which accounted for approximately 50% of the percentage of parasitaemia (Basir et al., 2012).

Anti-malarial Activity (4-Day Suppressive Test)

Male ICR mice (N = 42) were distributed into six groups of seven mice (n = 7), following simple randomization (Verhave *et al*., 2024) and each was given a different treatment (Ali *et al.*, 2017; Ali *et al.*, 2022; Hassan *et. al.* 2019). The groups of mice were the normal (saline 0.85%), *P. berghei* ANKA-infected (saline 0.85%), CQ-treated infected (10 mg/kg b.w.), and caffeine-treated infected (5, 10, and 20 mg/kg b.w.). For the malarial infection, the mice were injected with 0.2 mL of infected blood (2×10^7 pRBC) for four consecutive days (4-day suppressive test) prior to treatments.

Throughout the study, the basic parameters such as physical changes, body weight, and body temperature were measured and recorded daily. The body weight of the mice was measured using a weighing balance (Camry, Hong Kong), while the axillary temperature used a thermometer (medACCU, China). All mice were visually observed for signs of illness or behavioural changes such as lethargy, decreased locomotor activity, piloerection, and diarrhoea. Any indications of illness were categorized as absent (-), mild (+), moderate (++), or severe (+++) using an arbitrary scale. Survivability of the mice was recorded for 30 days. The thin blood smear was prepared every 2 days to observe the parasitaemia count.

Parasitaemia Measurement

The staining procedure was conducted based on Huang *et al.* (2015). A thin blood film was developed from the collected blood drop and stained with Geimsa's stain. The blinding procedure was implemented to minimize observer bias. The parasitaemia percentage was calculated in random order of slides from 5 random sites up to 1000 cells in total using a light microscope (Leica, Germany). This procedure was conducted to prevent systematic measurement bias that adheres to the principles outlined by the ARRIVE guidelines (Percie du Sert et al., 2020; Verhave et al., 2024).

The percentage of parasitaemia and chemosuppression were calculated using Peters and Robinson (1992) with slight modification.

$$\text{Percentage of Parasitaemia} = (\text{Number of parasitized RBC}/\text{Total number of RBC count}) \times 100$$

$$\text{Percentage of Chemosuppression} = ((\text{Parasitemia in negative control} - \text{Parasitemia in the study group})/\text{Parasitemia in negative control}) \times 100$$

The animal mean survival time was calculated using the formula below (Mekonnen, 2015):

Mean survival time (MST) = (Total number of mice survived/Total number of mice)

Cytokine Modulation Activity

The suppressive test was repeated with four groups of ten mice (n = 10): normal, infected mice, CQ-treated infected (10 mg/kg b.w.), and caffeine-treated infected (5 mg/kg b.w.). Upon completion of treatment (Day 5), all the mice were euthanized, and blood was drawn via cardiac puncture.

The pro-and anti-inflammatory cytokines were measured in the mice serum by means of enzyme-linked immunoassay (ELISA) method using the readily accessible kits. The pro-inflammatory cytokines includes TNF-α (ELABSCIENCE, USA; Catalog number: E-EL-M3063; Detection limit: 4.69 pg/mL), IFN-γ (ELABSCIENCE, USA; Catalog number: E-EL-M0048; Detection limit: 9.38 pg/mL) and IL-18 (MLB International, USA; Catalog number: 7625; Detection limit 25.0 pg/mL). The anti-inflammatory cytokines includes IL-4 (ELABSCIENCE, USA; Catalog number IL-4 : E-EL-M0043; Detection limit: 18.75 pg/mL) and IL-10 (ELABSCIENCE, USA; Catalog number IL-10 : E-EL-M0046; Detection limit: 9.38 pg/mL).

Statistical Analysis

The results were analysed using GraphPad Prism version 10.1.1 and the data presented as mean value +/- SEM. One-way analysis of variance (ANOVA) followed by Tukey's test as a single post-hoc test was used to compare the experiment group means with $p < 0.05$, $p < 0.01$ and $p < 0.001$ were considered statistically significant. Student's t-test and log-rank test were used to evaluate the data for Kaplan-Meier survival analysis.

RESULTS

Anti-malarial Activity (4-Day Suppressive Test)

Mice administered with 5 mg/kg b.w. of caffeine for four consecutive days following *P. berghei* ANKA infection resulted in significantly ($p < 0.05$) lowest parasite percentage of 8.38 ± 0.35% as compared to infected mice (Table 1; Fig. 1). Chemosuppressive effects of caffeine (5 mg/kg b.w.) demonstrated a significant ($p < 0.05$) increased percentage at 65.47 ± 1.42% as compared to infected mice (Table 2; Fig. 2). The median survival rate improved significantly in mice treated with 5 mg/kg b.w. of caffeine (17 Days) compared to infected mice (12 days) (Table 3; Fig. 3). The CQ-treated infected group exhibited the lowest parasite growth (1.22 ± 0.25%), the highest chemosuppression (94.99 ± 1.02), and the highest mean survival time (30 days) compared to other treatment groups. This is consistent with its function as an established anti-malarial reference drug (Zhou *et al.,* 2020).

Table 1**.** Parasitized red blood cells percentage (prbc %) of mice infected with *p. berghei* anka with or without caffeine treatment on day 4.

Group	Normal (n=7)	Infected mice (negative control) (n=7)	CQ-treated (10 mg /kg b.w.) (positive control) (n=7)	Caffeine-treated		
				(5 mg/kg b.w.) (n=7)	(10 mg/kg b.w.) (n=7)	(20 mg/kg b.w.) (n=7)
Mean PRBC percentage (%)	0	24.2 6 ± 5.11 [b-p < 0.001]	1.22 ± 0.25[a-p < 0.001]	8.38 ± 0.35[a-p < 0.001, b-p = 0.013]	14.90 ± 0.33[b-p = 0.003]	18.96 ± 0.49 [b-p = 0.01]

Mean PRBC was calculated on day-4 post infection. Data represent mean ± S.E.M for mean PRBC percentage (n = 7). [a] Significantly different comparison with infected control; [b] Significantly different comparison with CQ-treated; b.w., body weight; CQ, chloroquine; PRBC, parasitized red blood cells.

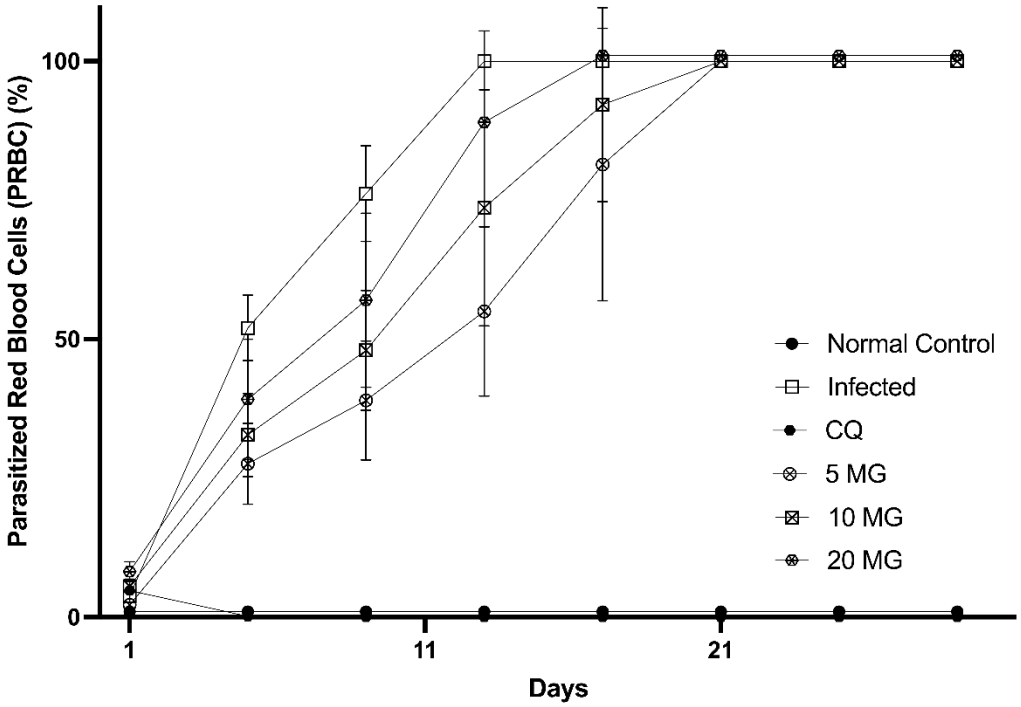


Fig. 1. Parasitized red blood cell (pRBC) percentage of mice infected with *P. berghei* ANKA with or without caffeine treatment throughout 30 days of monitoring. Data represent the pRBC percentage of normal (non-infected mice) (n = 7), negative control (nontreated *P. berghei* ANKA-infected mice) (n = 7), CQ-treated mice (n = 7), caffeine-treated (5 mg/kg b.w.) (n = 7), caffeine-treated (10 mg/kg b.w.) (n = 7) and caffeine-treated (20 mg/kg b.w.) (n = 7). A significant difference between groups was determined at *p < 0.05, **p < 0.01,***p < 0.001.

Table 2. Chemosuppression of mice infected with *P. berghei* anka with or without caffeine treatment on day 4

Group	Normal (n=7)	Infected mice (negative control) (n=7)	CQ-treated (10 mg /kg b.w.) (positive control) (n=7)	Caffeine-treated		
				(5 mg/kg b.w.) (n=7)	(10 mg/kg b.w.) (n=7)	(20 mg/kg b.w.) (n=7)
Chemo-suppression	100	0	94.99 ± 1.02[a-] P<0.001	65.47 ±1.42[a-] P<0.001, b-P<0.001	38.57±1.38[a-] P<0.001, b-P<0.001	21.85±2.01[a-] P<0.001, b-P<0.001

Chemo-suppression was calculated on day-4 post infection. Data represent mean ± S.E.M for chemosuppresion (n = 7); [a] Significantly different from infected control comparison; [b] Significantly different from CQ-treatment comparison; b.w., body weight; CQ, chloroquine.

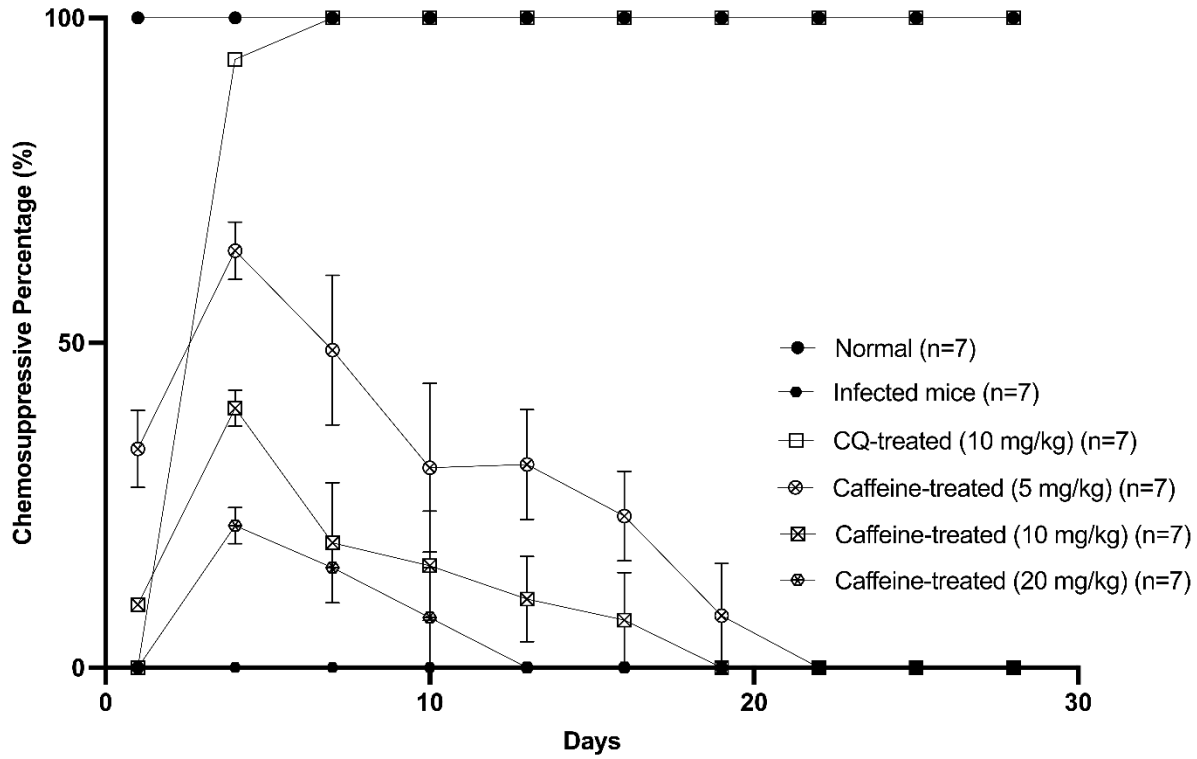


Fig. 2. Chemosuppressive of mice infected with *P. berghei* ANKA with or without caffeine treatment throughout 30 days of monitoring. Data represent chemosuppression of normal (non-infected mice) (n = 7), negative control (nontreated *P. berghei ANKA*-infected mice) (n = 7), CQ-treated mice (n = 7), caffeine-treated (5 mg/kg b.w.) (n = 7), caffeine-treated (10 mg/kg b.w.) (n = 7) and caffeine-treated (20 mg/kg b.w.) (n = 7). A significant difference between groups was determined at *p < 0.05, **p < 0.01,***p < 0.001.

Table 3. median survival time of mice infected with *p. berghei* anka with or without caffeine treatment throughout 30 days monitoring.

Group	Normal (n = 7)	Infected mice (negative control) (n = 7)	CQ-trated (10 mg/kg b.w.) (positive control) (n = 7)	Caffeine treated		
				(5 mg/kg b.w.)	(10 mg/kg b.w.)	(20 mg/kg b.w.)
Mean survival time	30 days [a- p] < 0.001	12 days [b – p] < 0.001	30 days [a – p] < 0.001	17 days [a – p; b = p < 0.001]	15 days [b-p < 0.001]	11 days [b – p] < 0.001

Median survival time was calculated on day-30 post infection. Data represent mean ± S.E.M for survival (n = 7).
[a] Significantly different comparison with infected control; [b] Significantly different comparison with CQ-treated; 4 b.w., body weight; CQ, chloroquine.

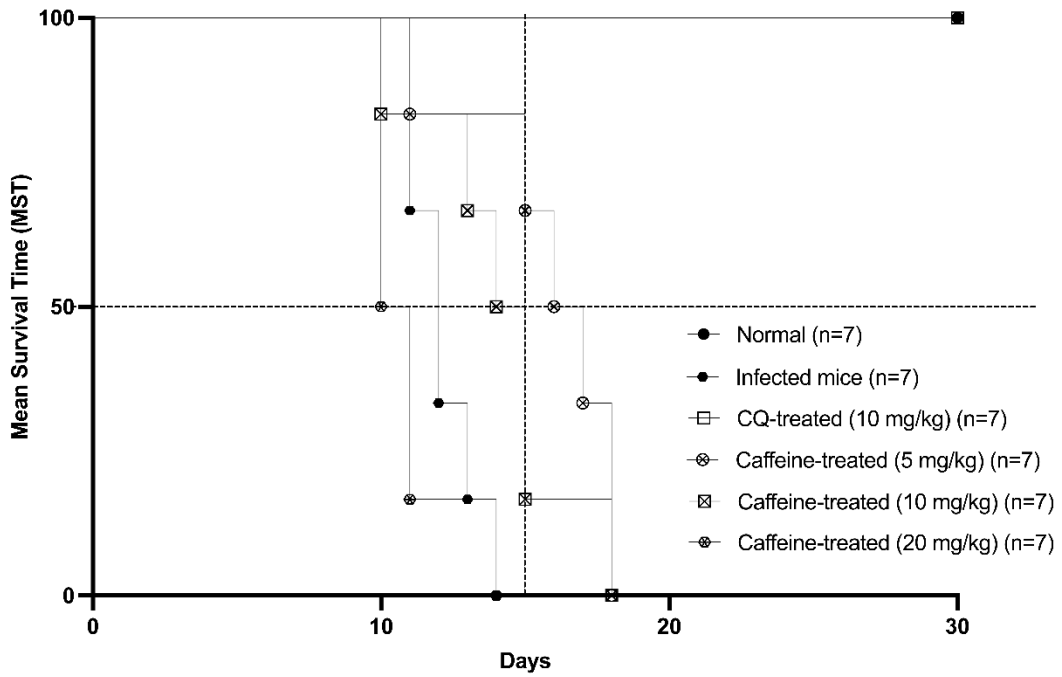


Fig. 3. Representative Kaplan-Meier survival curve of mice infected with *P. berghei* ANKA with or without caffeine treatment in a 30-day survivability test. Data represent survival of normal (non-infected mice) (n = 7), negative control (nontreated *P. berghei* ANKA-infected mice) (n = 7), CQ-treated mice (n = 7), caffeine-treated (5 mg/kg b.w.) (n = 7), caffeine-treated (10 mg/kg b.w.) (n = 7) and caffeine-treated (20 mg/kg b.w.) (n = 7). A significant difference between groups was determined at *$p < 0.05$, **$p < 0.01$,***$p < 0.001$.

The mice body weight in all groups was not significantly different ($p > 0.05$) when compared to normal mice (Fig. 4; Table 4). The body weight of the mice slightly increased for the normal group and CQ-treated infected group throughout 30 days of the survival test (Fig. 4). Mice from the caffeine-treated infected groups exhibited decreased body weight. However, they were not significantly reduced ($p > 0.05$) throughout the 30 days of monitoring after being given treatment for four consecutive days (Fig. 4). In addition, the body temperature of the mice in all groups was not significantly ($p > 0.05$) different from normal (Fig. 5; Table 5). Physical illness, including lethargy, decreased locomotor activity, and urine colour changes, were observed at 5 mg/kg b.w. of caffeine much later compared to other caffeine-treated infected groups (Appendix 1).

Table 4. Mean body weight of mice infected with *p. berghei* anka with or without caffeine treatment on day 4

Group	Normal (n=7)	Infected mice (negative control) (n=7)	CQ-treated (10 mg /kg b.w.) (positive control) (n=7)	Caffeine treated		
				(5 mg/kg b.w.) (n=7)	(10 mg/kg b.w.) (n=7)	(20 mg/kg b.w.) (n=7)
Body Weight (grams)	29.83± 1.47 [n.d.]	29.17± 1.17 [n.d.]	28.83 ± 2.86 [n.d.]	30.17± 4.4 [n.d.]	31.17± 3.251 [n.d.]	29.5 ± 5.86 [n.d.]

Body weight was calculated on day-4 post infection. Data represent mean ± S.E.M for body weight (n = 7).

[a] Significantly different comparison with infected control

[b] Significantly different comparison with CQ-treated

[n.d.] No significantly different comparison with control groups

b.w., body weight; CQ, chloroquine.

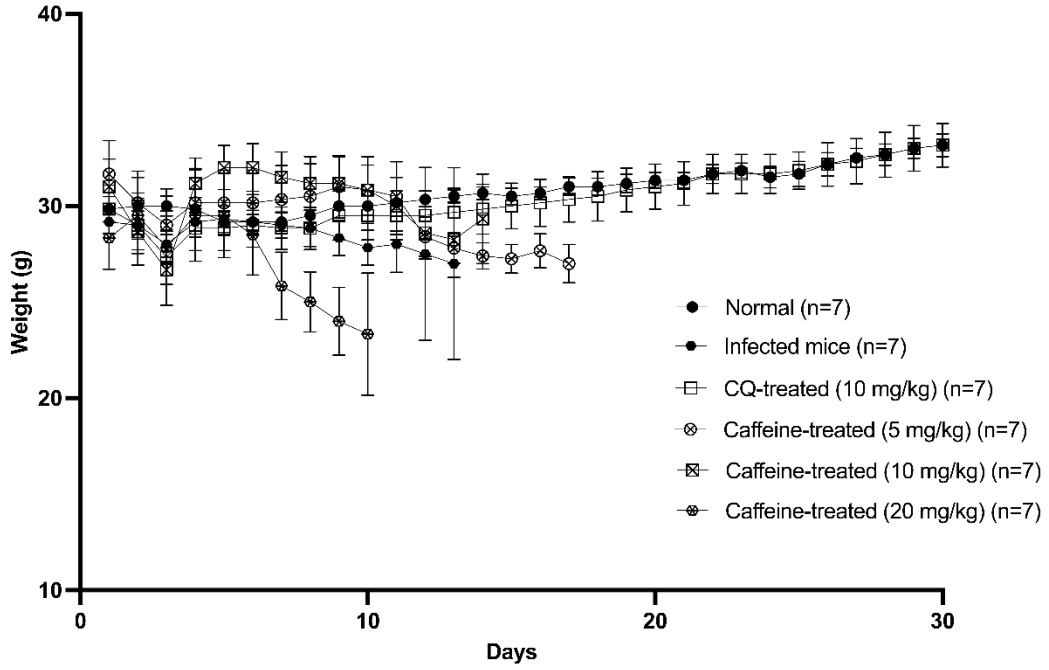


Fig. 4. Body weight of mice infected with *P. berghei* ANKA with or without caffeine treatment throughout 30 days of monitoring. Data represent survival of normal (non-infected mice) (n = 7), negative control (nontreated *P. berghei* ANKA-infected mice) (n = 7), CQ-treated mice (n = 7), caffeine-treated (5 mg/kg b.w.) (n = 7), caffeine-treated (10 mg/kg b.w.) (n = 7) and caffeine-treated (20 mg/kg b.w.) (n = 7). A significant difference between groups was determined at *p < 0.05, **p < 0.01,***p < 0.001.

Table 5: Mean body temperature of mice infected with *p. berghei* anka with or without caffeine treatment on day 4

Group	Normal (n=7)	Infected mice (negative control) (n=7)	CQ-treated (10 mg /kg b.w.) (positive control) (n=7)	Caffeine treated		
				(5 mg/kg b.w.) (n=7)	(10 mg/kg b.w.) (n=7)	(20 mg/kg b.w.) (n=7)
Body Temperature (°C)	37.1 ± 0.17 [n.d.]	37.08 ± 0.18 [n.d.]	37.12 ± 0.21 [n.d.]	37.02 ± 0.23 [n.d.]	36.85± 0.3 [n.d.]	36.72 ± 0.33 [n.d.]

Body temperature was calculated on day-4 post infection. Data represent mean ± S.E.M for body temperature (n = 7).

[a] Significantly different comparison with infected control

[b] Significantly different comparison with CQ-treated

[n.d.] No significantly different comparison with control groups

b.w., body weight; CQ, chloroquine; °C, degree Celsius.

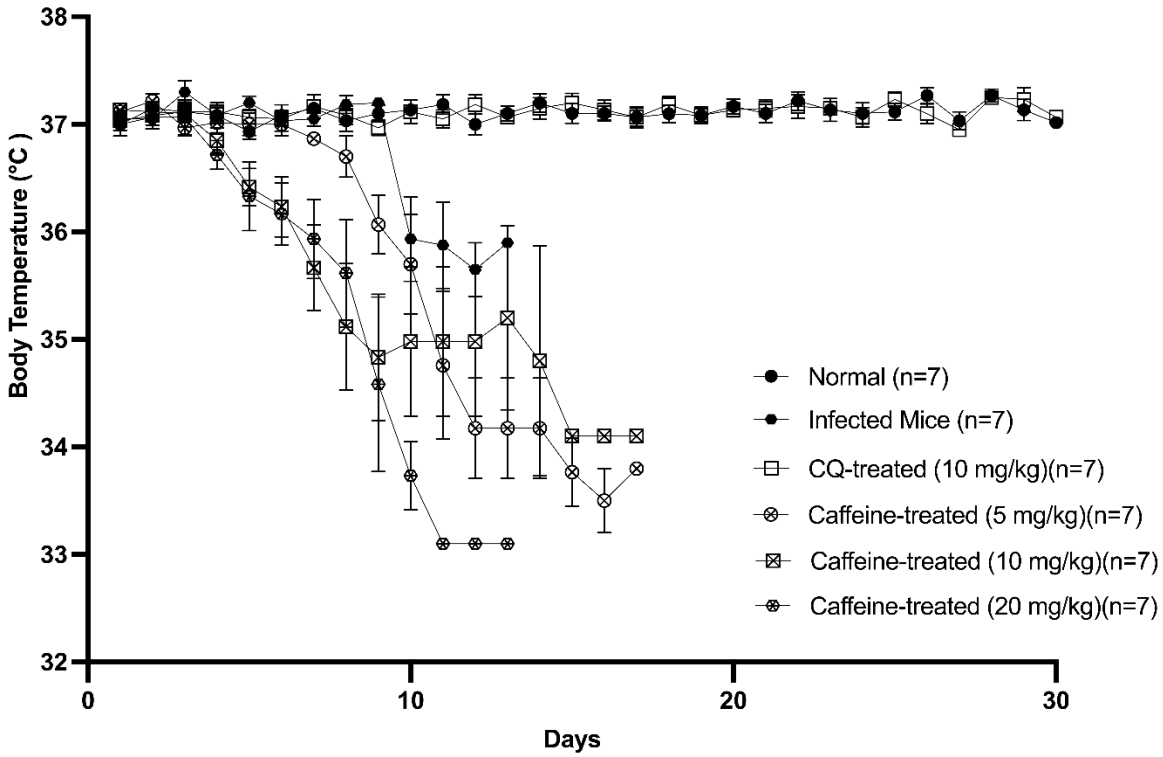
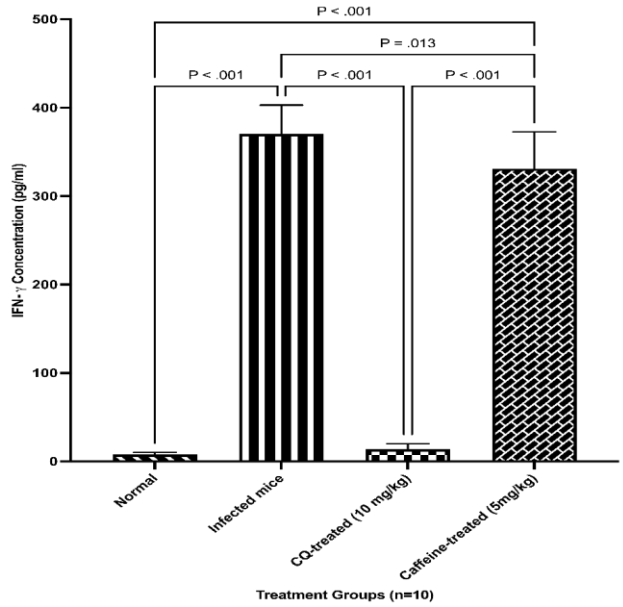


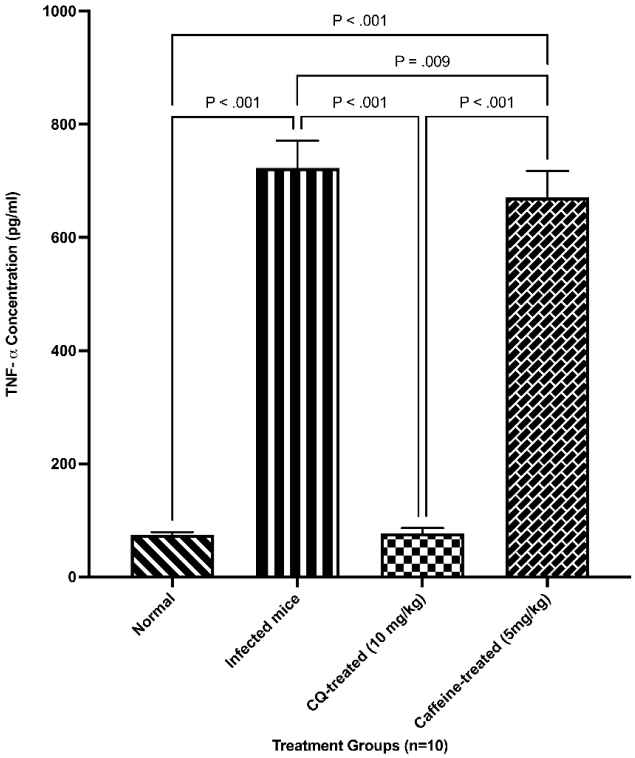
Fig. 5. Body temperature of mice infected with *P. berghei* ANKA with or without caffeine treatment throughout 30 days of monitoring. Data represent survival of normal (non-infected mice) (n = 7), negative control (nontreated *P. berghei* ANKA-infected mice) (n = 7), CQ-treated mice (n = 7), caffeine-treated (5 mg/kg b.w.) (n = 7), caffeine-treated (10 mg/kg b.w.) (n = 7)

and caffeine-treated (20 mg/kg b.w.) (n = 7). A significant difference between groups was determined at *p < 0.05, **p < 0.01,***p < 0.001.

Cytokine-Modulation Activity

The serum cytokine levels of the mice were examined for modulations of cytokine activities. The present result in Fig. 6, Fig. 7, and Table 6 indicates that there was an increase in both pro-and anti-inflammatory cytokines on day 4 after *P. berghei* ANKA infection. Parasite infection increased the level of the pro- and anti-inflammatory cytokines of TNF-α, IL-18, IFN-γ, IL-4, and IL-10 at 9.70-, 4.89-, 47.89-, 2.77-, and 2.75 folds, respectively, compared to normal mice. CQ exhibited reductions in TNF- α, IL-18, IFN-γ, IL-4, and IL-10 by 9.41-, 4.37-, 27.01-,1.2-, and 2.39-folds, respectively, compared to infected mice (Table 6). The pro- and anti-inflammatory cytokines of TNF-α, IFN-γ, IL-18, and IL-10 were significantly reduced (by 1.08-, 1.12-, 1.12-, and 1.15-folds respectively) in the serum after four days of caffeine treatment at 5 mg/kg b.w. as compared to the infected mice (Figure 6, Figure 7, and Table 7). IL-4 cytokine level did not exhibit any significant difference when treated with caffeine compared to the infected mice (1.09 folds). Levels of pro-and anti-inflammatory cytokines of TNF-α, IL-18, IFN-γ, and IL-10 were significantly reduced with CQ treatment. CQ was able to enhance parasite infection by reducing inflammatory cytokine production since it can eradicate parasites in the initial phase of infection. Like quinine and mefloquine, CQ can directly affect the immune system (Kwiatkowski and Bate, 1995; Picot *et al.*, 1993). Although CQ at 10 mg/kg b.w. can significantly reduce IL-4 cytokine level (2.3-fold), the level is not as low as in normal mice (Table 6). The same pattern was exhibited by caffeine treatment, which did not significantly reduce IL-4 cytokine level as compared to infected mice.


P < .001
P = .013
P < .001
P < .001
P < .001
500
400
300
200
100
0
IFN- γ Concentration (pg/ml)
Normal
Infected mice
CQ-treated (10 mg/kg)
Caffeine-treated (5mg/kg)
Treatment Groups (n=10)


P < .001
P = .009
P < .001
P < .001
P < .001
1000
800
600
400
200
0
TNF- α Concentration (pg/ml)
Normal
Infected mice
CQ-treated (10 mg/kg)
Caffeine-treated (5mg/kg)
Treatment Groups (n=10)

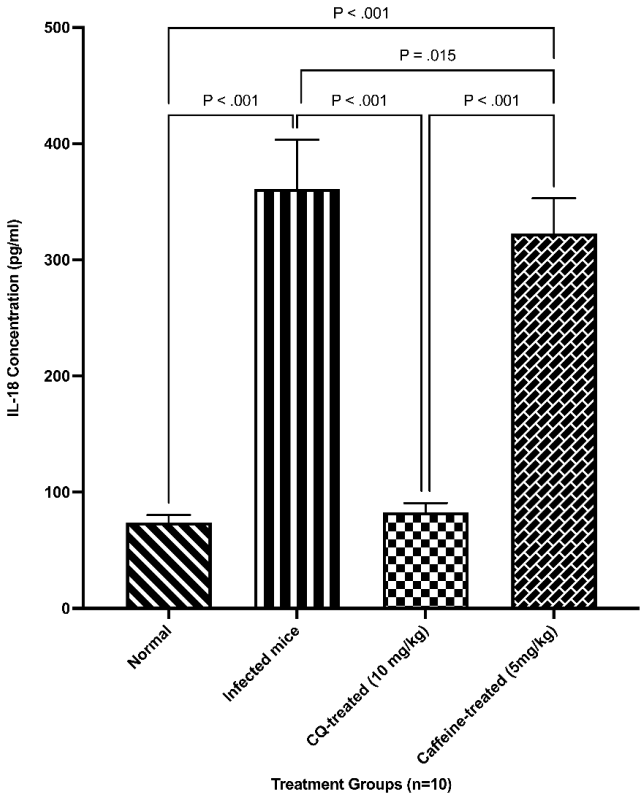


Fig. 6. Serum concentration of pro-inflammatory cytokines of (A) TNF-α, (B) IFN-γ and (C) IL-18 with Normal, Infected mice, CQ treated (10 mg/kg b.w.) and caffeine treated (5 mg/kg b.w.) on day-4 post-infection. Data represent mean ± S.E.M of Normal (n = 10)., Infected mice (n = 10)., CQ treated (10 mg/kg b.w.) (n = 10)., and caffeine treated (5 mg/kg b.w.) mice (n = 10). A significant difference between groups was determined at *p < 0.05, **p < 0.01,***p < 0.001. CQ, chloroquine; IFN, Interferon; IL, interleukin; TNF, Tumour Necrosis Factor.

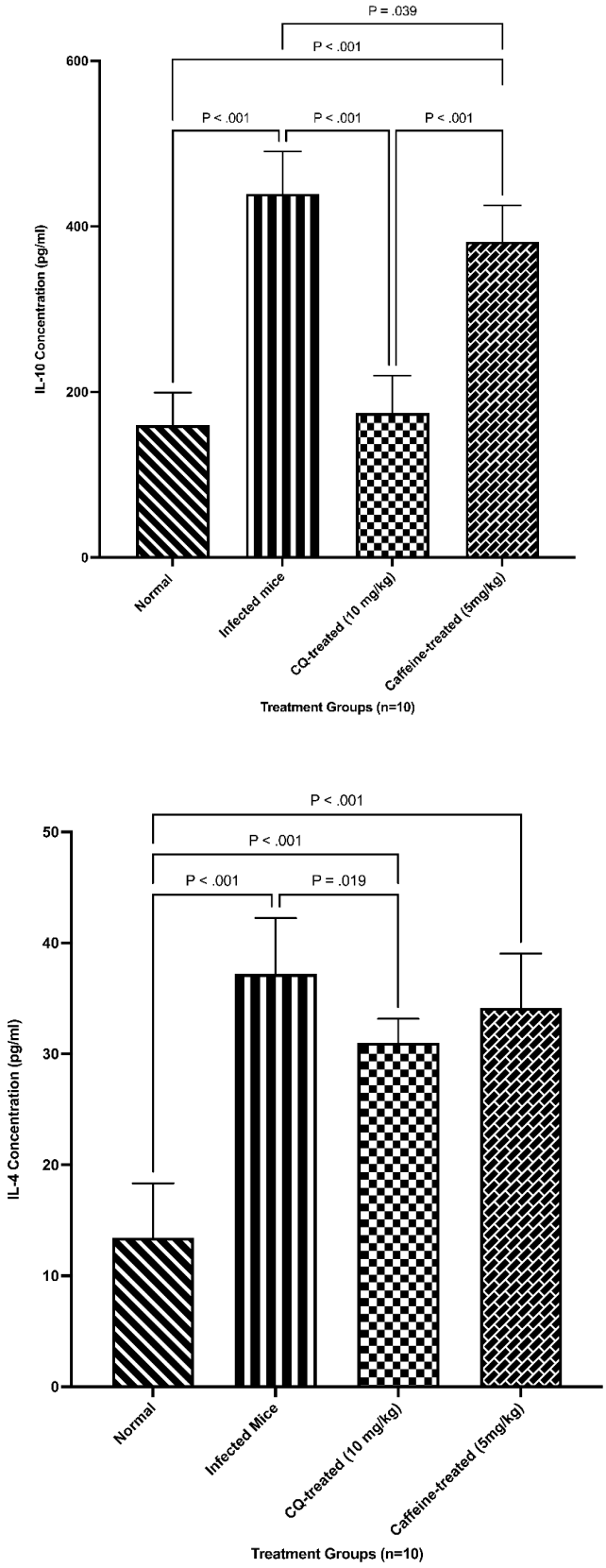


Fig. 7. Serum concentration of anti-inflammatory cytokines of (A) IL-4 and (B) IL-10 with Normal, Infected mice, CQ treated (10 mg/kg b.w.) and caffeine treated (5 mg/kg b.w.) on day-

4 post-infection. Data represent mean ± S.E.M of Normal(n = 10), Infected mice (n = 10)., CQ treated (10 mg/kg b.w.) (n = 10), and caffeine treated (5 mg/kg b.w.) mice (n = 10). A significant difference between groups was determined at *p < 0.05, **p < 0.01,***p < 0.001. CQ, chloroquine; IL, interleukin.

Table 6. Inflammatory cytokines level of mice infected with *P. berghei* anka treated with or without caffeine treatment.

Cytokines		Groups			
		Normal (n=10)	Infected mice (negative control) (n=10)	CQ-treated (10 mg /kg b.w.) (positive control) (n=10)	Caffeine (5 mg/kg b.w.) (n=10)
Pro-inflammatory cytokine	Tumour nuclear factor alpha (TNF- α)	74.54 ± 1.48[a-P<0.001]	722.8 ± 15.27[b-P<0.001]	76.78 ± 3.08 [a-P<0.001]	670.6 ± 14.82[a-P=0.009,b-P<0.001]
	Interferon Gamma (IFN- γ)	7.733 ± 0.74[a-P<0.001]	370.3 ± 10.30[b-P<0.001]	13.71 ± 2.04[a- P<0.001]	331.3 ± 13.09[a-P=0.013,b-P<0.001]
	Interleukin -18 (IL-18)	73.85 ± 2.03[a-P<0.001]	361.0 ± 13.40[b-P<0.001]	82.59 ± 2.57[a-P<0.001]	322.4 ± 9.63[a-P=0.015, b-P<0.001]
Anti-inflammatory cytokine	Interleukin -4 (IL-4)	13.43 ± 1.55[a-P<0.001]	37.22 ± 1.59[b-P=0.019]	31.00 ± 0.69 [a-P=0.019]	34.16 ± 1.55 [n.d.]
	Interleukin -10 (IL-10)	159.7 ± 12.49[a-P<0.001]	439.6 ± 16.10[b-P<0.001]	174.7 ± 14.29[a-P<0.001]	381.5 ± 13.94[a-P=0.039, b-P<0.001]

Body temperature was calculated on day-4 post infection. Data represent mean ± S.E.M for cytokine level (n = 10).

[a] Significantly different comparison with infected control

[b] Significantly different comparison with CQ-treated

[n.d.] No significantly different comparison with control groups

b.w., body weight; CQ, chloroquine; IFN, Interferon, IL, interleukin; TNF, Tumour Necrosis Factor.

DISCUSSION

Caffeine is known for its function as a stimulant for the central nervous system in increasing focus and alertness and elevating fatigue (Barcelos *et al*., 2020). Caffeine is commonly taken in the form of drinks such as coffee or tea (Pohanka, 2015). In a previous report, caffeine demonstrated its ability as an anti-plasmodial activity through an *in vitro* study where a high inhibition percentage of caffeine pyrazolines was identified against *P. falciparum* (Insuasty *et al*., 2015). It is also worth mentioning that caffeine has several properties that have been reported. This includes anti-inflammatory, antioxidant, and neuroprotective, especially for the blood-brain barrier, particularly for neurodegenerative diseases (Azam *et al*., 2003; Barcelos *et al.*, 2020; Yacoubi *et al*., 2000; Kolahdouzan and Hamadeh, 2017).

The animal model used in this study mimic the injury of cerebral malaria. The similarity of the neurological conditions of neurodegenerative diseases with cerebral malaria, such as brain ischemia-like syndrome, excitotoxicity, and inflammation, suggested that treatment with caffeine in cerebral malaria infection may also be associated with the A2AR receptor

(Kolahdouzan and Hamadeh, 2017). Previous studies reported that caffeine protects against neurodegenerative disease by binding to the A2AR pathway and acting as an antagonist to A2AR receptors (Chen *et al.,* 2010). Yacoubi *et al*. (2000) also strengthened the fact of A2AR participation caused by the stimulant effects of caffeine at 6.25 mg/kg b.w. in locomotor stimulatory effect in CD1 mice (Yacoubi *et al*., 2000). The study also stated that when the concentration of caffeine increases, the inhibition of the A2AR receptor decreases (Yacoubi *et al.*, 2000).

Recently, plants have been used as immunomodulatory agents where natural resources have been proven to be safe and effective alternatives (Alhazmi *et al*., 2021). Immunomodulatory strategies are considered adjunctive therapy when the elimination of parasites cannot be achieved through antiparasitic approaches to reduce disease severity and burden (Hassan *et al.*, 2022). Caffeine is thus a potential molecule from plant-based immunomodulators to address parasite infection as an adjunctive therapy (Kovács *et al.*, 2021). Our findings reveal that caffeine administration into *P. berghei* ANKA-infected ICR mice effectively suppressed parasitaemia growth in red blood cells and improved the survivability of infected mice, providing proof that caffeine has good anti-malarial properties. Caffeine administration at a low dose (5 mg/kg b.w.) resulted in a significant reduction ($p < 0.05$) of parasite percentage, a significant increase in chemosuppressive percentage, and a significantly prolonged survivability of malaria-infected mice.

The result also suggested that as the concentration of caffeine increases, the chemosuppressive percentage and the survivability of infected mice decrease. Similar findings were identified from a previous study using *P. berghei* ANKA in ICR mice but with a different compound, quercetin (Ali *et al*., 2021). The chemosuppression percentage of quercetin at 15, 25, and 50 mg/kg b.w. were recorded as 36.1 ± 5.7, 24.9 ± 3.8, and 24.3 ± 1.8%, respectively (Ali et al., 2021). Another study using andrographolide treatment in *P. berghei* NK65-infected ICR mice also reported similar findings where at 5, 15, and 45 mg/kg b.w. showed chemosuppression at 60.17 ± 2.12%, 58.96 ± 4.75%, and 43.18 ± 6.28%, respectively (Hassan *et al*., 2019). Our result was in line with the above-mentioned studies, which caffeine at 5, 10, and 20 mg/kg b.w. exhibited chemosuppression percentage of 64.18 ± 9.17, 39.93 ± 5.63, and 21.86 ± 5.57%, respectively. Similar trends are also identified for the survival time as the concentration of caffeine (5, 10, and 20 mg/kg b.w.) increases, and the mean survival times of mice decrease (17, 15, and 11 days). Quercetin treatment at 15, 25 and 50 mg/kg b.w. resulted at 10, 9, and 9 days and andrographolide at 5, 15, and 45 mg/kg b.w. recorded mean survival times as 19, 17, and 14 days in malaria-infected mice (Ali *et al*., 2021; Hassan *et al.*, 2019).

Caffeine has anti-obesity properties where caffeine is able to undergo cellular energy metabolism and has an antagonist effect towards adenosine receptors, which results in the

inhibition of adenylyl cyclase enzyme and upregulation of cyclic adenosine monophosphate (cAMP) levels (A2AR pathway) (de Souza *et al*., 2021). Free fatty acids from the lipolysis of the cycle are then used by *Plasmodium spp*. led increases of parasite growth (Maier and van Ooij, 2022). This is due to the fact that *Plasmodium spp.* depends on the host to produce fatty acids (Jayabalasingham *et al*., 2010). However, the loss of body weight and body temperature were insignificant in all treatment groups compared to infected mice, as reported in the cerebral malaria murine model study (Ali *et al*., 2022; Basir *et al*., 2012).

A previous study identified that caffeine treatment at 5, 10, and 20 mg/kg b.w. given together with olive oil resulted in a good chemosuppressive percentage exhibited 79.09%, 83.64%, and 87.27%, respectively (Fadare *et al*., 2024). Our study revealed lower chemosuppressive results than the previous study (Fadare *et al.,* 2024). Due to the pathway of drug delivery via oral gavage and the bypass lymphatic pathway, it is possible that the drug delivery method and dilution with distilled water are the reasons for the contradiction with our findings (Yan *et al.,* 2023). The study elucidated absorption using a lipid-based drug delivery system where it bypasses the first metabolic pathway into the lymphatic system (Yan *et al.*, 2023). In addition, the study also stated that different dose formulations have different absorption rates where absorption of caffeine dissolved in distilled water is slower than caffeine dissolved in olive oil (Fadare *et al.*, 2024).

In addition, caffeine (5 mg/kg b.w.) reduced pro-inflammatory cytokines (TNF-α, IFN-γ, IL-18) and anti-inflammatory cytokines (IL-4 and IL-10) levels as compared to malaria-infected mice. Our results exhibited a significant reduction ($p < 0.05$) of pro-inflammatory cytokines level (TNF-α and IFN-γ), corroborating previous findings with quercetin and andrographolide (Ali *et al*., 2021; Hassan *et al*., 2019). However, anti-inflammatory cytokine levels were significantly lower than infected mice for IL-10, and there was no significant difference in IL-4 levels in caffeine-treated malarial mice. Furthermore, IL-4 revealed a reduction when given treatment with CQ compared to infected mice, but it is not significant, similar to a previous study (Ali *et al.*, 2021). Another study by Sudi *et al*. (2018) also indicated no significant reduction of IL-4 cytokine levels when given CQ treatment.

Findings from previous studies have revealed the possible role played by each cytokine in the pathogenesis of malaria (Bhatt *et al.*, 2021; Angulo and Fresno, 2002; Suhaini *et al*., 2015; Wong *et al*., 2015). Caffeine has also been reported to reduce cytokine production (IFN-γ, TNF-α, IL-10, and IL-18) levels in *in vitro* studies (Pohanka, 2015). A previous study of virulent *Listeria monocytogenes* in Swiss mice showed that caffeine did not exhibit any changes in IL-10 plasma level after treatment via i.p. injection. The study explains that the cause could be the route of administration (De Alcântara Almeida *et al*., 2021).

As caffeine has a biphasic effect, treatment with caffeine would not reduce pro-inflammatory cytokine levels as low as CQ treatment. Pro-inflammatory cytokines level of caffeine treatment (TNF-α, IFN-γ, IL-18) decreased slightly (1.07-, 1.11- and 1.12-fold) as compared to infected control mice. Therefore, during malarial infection, the concentration increases from 10 to 20 mg/kg b.w. of caffeine, resulting in excessive pro-inflammatory cytokine levels, which results in greater tissue damage with malarial presence. Thus, this causes organs to be inflamed, resulting in chronic infection. This is proven by Otha *et al*. (2007), who reported that 10 mg/kg b.w. of caffeine showed significant liver damage and 20 mg/kg b.w. showed a higher reading of liver damage in acute liver study. However, caffeine at 100 mg/kg b.w. did not show any liver damage in the acute liver study (Ohta *et al.*, 2007). The investigation of the model of ischemia-reperfusion (IR) by inhibiting A2AR shows the reduction of pERK activation and glutamate protein levels and downstream inflammatory responses (Mohamed *et al*., 2016). The levels of pro-inflammatory biomarkers such as NF-κB, TNF-α, IL-6, and prostaglandin E2 were lowered, while the anti-inflammatory biomarker IL-10 was increased similarly to our control group (Mohamed *et al*., 2016).

The A2A adenosine receptor (A2AR) activates with the binding of adenosine to the receptor triggering pro-inflammatory signalling pathway (Kolahdouzan & Hamadeh, 2017). Once activated, A2AR triggers Gs-coupled signalling that increases cAMP and activates protein kinase A (PKA), which enhances calcium influx and glutamate release, leading to activation of the NF-κB pathway and production of inflammatory cytokines such as TNF-α and IL-6 (Kolahdouzan & Hamadeh, 2017). Caffeine acts as a competitive antagonist at A2AR, blocking adenosine from binding (Kolahdouzan & Hamadeh, 2017). This prevents the rise in cAMP and PKA activity, reduces calcium and glutamate-driven inflammation, and inhibits NF-κB activation. Ultimately, the cascades lowered the release of inflammatory cytokines and produced an overall anti-inflammatory and neuroprotective effects (Kolahdouzan & Hamadeh, 2017).

Concentration treatments that are selected in the experiment are low-to-moderate doses of caffeine (5, 10 and 20 mg/kg b.w.) and are widely used in rodent models evaluating neuroprotective, anti-inflammatory, and infection-related responses. Higher concentration of caffeine at 30 mg/kg b.w. is not chosen due to the fact that there is excessive systemic toxicity (Chen *et al.*, 2010).

The result shows that caffeine exhibits a dose-dependent paradox in which increasing concentration can ultimately decrease its inhibitory efficacy. At the molecular level, caffeine acts as a competitive antagonist at adenosine receptor particularly at A_2A receptor (A_2AR) in the brain where caffeine competing with adenosine for the same binding site (Chen et al., 2020; Borea et al., 2018). At low or acute doses, caffeine effectively binds to A_2AR without activating it, thereby blocking adenosine and pro-inflammatory signaling pathways (Chen et

al., 2020). Thus, the blockade suppresses downstream NF-κB activation through cAMP–PKA signaling modulation, resulting in lower inflammatory cytokine output (Liu et al., 2024). In this context, caffeine produces high inhibitory efficacy and reduced inflammatory signaling (Barcelos et al., 2020).

However, under chronic or high-dose exposure, the brain and peripheral tissues activate homeostatic feedback mechanisms. Prolonged A_2AR blockade by caffeine induces adaptive upregulation of adenosine receptors, increasing A_2AR density and thereby reducing caffeine's relative blocking efficiency (de Souza Gonçalves et al., 2017; Eichwald et al., 2023). With more A_2AR available, caffeine must compete against higher adenosine levels for a larger receptor pool, diminishing its antagonistic efficacy and contributing to pharmacological tolerance (Chen et al., 2020). As A_2AR signalling regains influence, NF-κB–mediated inflammatory pathways can become reactivated, promoting increased cytokine production compared with the low-concentration (Liu et al., 2024; Marcinek et al., 2024). Thus, these may lead to higher inflammatory cytokines production.

Taken together, findings demonstrate that the effective dose of caffeine can suppress parasite growth and modulate the cytokines levels in malaria-infected ICR mice. The role of caffeine in enhancing the immune response against *P. berghei* ANKA, particularly its effects on pro-inflammatory cytokine productions, demonstrated that caffeine interactions with cells are an area for further exploration. Additionally, the study raises the significance of investigating the pharmacodynamics and potential mechanisms of caffeine's action against malarial parasites. Theoretically, caffeine at 5 mg/kg b.w. can inhibit A2AR, which reduces the production of inflammatory cytokines and slows down malarial infection growth.

This study acknowledges several limitations. Histopathological analyses of the brain, liver, and spleen were not performed to compare infected tissues with treated animals. Such analyses would have provided stronger evidence regarding the potential of caffeine to improve cerebral malaria conditions. Additionally, oxidative stress biomarkers, including malondialdehyde (MDA) and reduced glutathione (GSH), were not evaluated. Another limitation is the use of only male mice. Female mice are not used due to occurrence in hormonal imbalance such as estrogen which increased the oxidative stress, influence malarial parasite growth and lead to cytokine storm, potentially affecting the consistency of results within the experimental groups (Wu *et. al.*, 2025).

Further investigation into the pharmacodynamic profile of caffeine in the context of malaria treatment is also recommended to better understand its mechanism of action and therapeutic potential. Future studies should explore the potential of combination therapies involving caffeine and standard antimalarial drugs, such as artesunate or CQ, to evaluate possible synergistic effects.

CONCLUSION

The study has shown that caffeine possesses anti-malarial properties and is able to modulate inflammatory cytokine levels. Treatment with caffeine at 5 mg/kg b.w. in *P. berghei* ANKA-infected ICR mice was able to slow down parasite growth as compared to 10 and 20 mg/kg b.w. of caffeine treatment. Notably, the low dose caffeine exhibited a good chemosuppressive effect (> 60%) and was able to significantly prolong the survival time of malaria-infected ICR mice (17 days). Furthermore, caffeine at 5 mg/kg b.w. modulated cytokine levels of the pro-inflammatory (TNF- α, IFN-γ, and IL-18) and anti-inflammatory (IL-4 and IL-10) cytokines. This results in the anti-malarial and cytokine-modulating properties in ICR mice. The author also plans to identify the mechanism of caffeine, i.e., whether caffeine is able to block the A2AR pathway.

AUTHORSHIP CONFIRMATION/CONTRIBUTION STATEMENT

Author 1: writing-original draft, review and editing, conceptualization, formal analysis, methodology, investigation . Author 2: resources, supervision, writing–review. Author 3: supervision, writing–review. Author 4: resources, supervision, writing–review. Author 5: supervision, writing–review. Author 6: writing-review and editing, conceptualization, validation, supervision and funding.

AUTHOR(S') DISCLOSURE (CONFLICT OF INTEREST) STATEMENT(S)

The authors declare no potential conflicts of interests.

FUNDING STATEMENT, EVEN WHEN NOT APPLICABLE

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APPENDIX

Appendix 1. Physical signs throughout 30 days monitoring.

Physical Signs	Category	Days Appeared					
		Normal	Infected mice (negative control)	CQ-treated (10 mg /kg b.w.) (positive control)	Caffeine-treated		
					5 mg/kg b.w.	10 mg/kg b.w.	20 mg/kg b.w.
Lethargic	Absent	Day 30	Day 2	Day 30	Day 8	Day 3	Day 3
	Mild	0	Day 3	0	Day 9	Day 4	Day 4
	Moderate	0	Day 4	0	Day 11	Day 5	Day 5
	Severe	0	Day 5	0	Day 15	Day 8	Day 6
Piloerection	Absent	Day 30	Day 2	Day 30	Day 5	Day 2	Day 2
	Mild	0	Day 3	0	Day 6	Day 3	Day 3
	Moderate	0	Day 4	0	Day 8	Day 4	Day 4
	Severe	0	Day 5	0	Day 12	Day 5	Day 5
Reduced Locomotor Activity	Absent	Day 30	Day 2	Day 30	Day 6	Day 3	Day 2
	Mild	0	Day 3	0	Day 7	Day 4	Day 3
	Moderate	0	Day 4	0	Day 9	Day 5	Day 4
	Severe	0	Day 5	0	Day 11	Day 5	Day 5
Urine Colour	Absent	Day 30	Day 2	Day 30	Day 8	Day 4	Day 3
	Mild	0	Day 3	0	Day 9	Day 5	Day 4
	Moderate	0	Day 4	0	Day 11	Day 6	Day 5
	Severe	0	Day 5	0	Day 14	Day 9	Day 7

Physical signs changes was observed throughout 30 days. Data represent days appearance on mice. CQ, chloroquine.