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Nanoparticles of beetroot extract: A potential antimalarial adjuvant in *Plasmodium berghei*-infected mice

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ABSTRACT

Malaria disease is caused by a variant of mosquitoes that leads to transmission in humans. This study aimed to investigate the effects of beetroot extract (NBE) nanoparticles on parasitemia index and ICAM-1 levels in mice infected with *Plasmodium berghei*. Thirty male mice were randomly divided into six groups including H= baseline (normal/healthy), NC= *P. berghei* inoculation, PC= *P. berghei* inoculation treated with artemisinin, T1= *P. berghei* inoculation treated with artemisinin and NBE (50 mg/kg), T2= *P. berghei* inoculation treated with artemisinin and NBE (100 mg/kg), and T3= *P. berghei* inoculation treated with artemisinin and NBE (200 mg/kg). Parasitemia index in groups T2 (13.1%) and T3 (12.5 %) were significantly lower compared to groups NC (36 %) and PC (16.2 %). Furthermore, ICAM-1 levels in groups T2 (145.72 ng/ml) and T3 (151.24 ng/ml) were significantly lower compared to groups NC (319.17 ng/ml) and PC (241.93 ng/ml). The findings of the study suggest that nanoparticles of beetroot extract could have potential as an anti-inflammatory and antimalarial adjuvant for malaria infection.

INTRODUCTION

Malaria is an infectious disease caused by the parasitic organism of the *Plasmodium* genus. Malaria infection is a global concern since the mortality and morbidity rates are still quite high. The high mortality rate of malaria infections is due to severe pathological occurrence with organ failure and severe malaria, especially in malaria-infected by *Plasmodium falciparum* [1,2]. The pathogenesis of severe malaria is influenced by the increased expression of intercellular adhesion molecule-1 (ICAM-1) in blood vessel endothelial cells. The immune response to malaria infection causes excessive production of pro-inflammatory cytokines, thus triggering ICAM-1 expression on the endothelium. Excessive ICAM-1 causes the sequestration of infected erythrocytes (PRBC) in the microvasculature and increases the adhesion of PRBC to endothelial cells, which causes vascular obstruction and decreases venous blood flow. These incidences lead to multiple organ failures [3–5].

ICAM-1 in soluble form (sICAM-1) increases serum levels in response to increased pro-inflammatory cytokines due to malaria disease. The concentration of ICAM-1 in the serum of malaria patients reflects in increasing the inflammatory response and activation of endothelial cells with the release of soluble ICAM-1[6]. The results of the previous studies indicated that serum ICAM-1 levels increased in patients with severe or fatal malaria, suggesting that serum ICAM-1 level is an indicator related to the severity of malaria [7]. This research uses Balb/c strain mice inoculated with ANKA

strain of *Plasmodium berghei* which may cause malaria in rodents such as rats and mice. Molecular analysis shows similarities between *P. falciparum* and *P. berghei* ANKA, thus *P. berghei* ANKA is often used as a model in experimental animals for malaria research.

Beetroot is a vegetable crop belonging to the Amaranthaceae family and contains the active ingredient betalain. The beetroot betalain consists of two main components, betacyanin, and betaxanthin, which have anti-inflammatory potential in previous studies [8,9]. Betalains, derived from betaxanthin, have a role in modulating the production of pro-inflammatory cytokines, which influence the regulation of ICAM-1 expression [8,9]. However, the role of beetroot ingredients in malaria treatment is not well understood. In addition, the use of natural ingredients as adjuvant therapy still causes several limitations, such as active substances that are difficult to penetrate cell membranes, thus reducing their effectiveness. To achieve the expected therapeutic effect, the optimum dose and treatment duration need to be fine-tuned. Following the solution of these points can increase the effectiveness and speed of absorption of these natural ingredients needed to achieve therapeutic effects. Nanomedicine technology has recently been widely used to increase the effectiveness of drug absorption in smaller doses, thereby minimizing side effects but achieving the desired therapeutic effect [10,11]. Chitosan can be formulated as polymeric nanoparticles for oral administration, as it can enhance penetration by opening the tight junction epithelium and interacting with mucus by forming ionic or hydrogen bonds [12]. Chitosan nanoparticles are biocompatible, biodegradable, and non-toxic, preventing enzymatic drug degradation and reducing damage to target tissue or cells [13,14]. Additionally, the slow biodegradation of chitosan nanoparticles ensures its potential regulation of drug release. The positive surface charge of chitosan nanoparticles makes it a stable drug carrier for active compounds within the body until it reach the target cells [15]. Integrating chitosan nanoparticles into herbal medicine signifies a promising advancement in the development of drug carriers. The purpose of this study is to evaluate the antimalaria and anti-inflammatory effects of beetroot extract nanoparticle supplementation in mice infected with *P. berghei* and treated with artemisinin.

MATERIALS AND METHODS

Ethical statement

The research was conducted in the Parasitology and Pharmacology laboratory of FKKMK, Gajahmada University, Yogyakarta. The study was approved by The Health Research Ethics Committee Faculty of Medicine Diponegoro University (No: 13/EC/H/FK-UNDIP/I/2023).

Collection and extraction of beetroot

Beetroots (*Beta vulgaris* L.) were collected from Kopeng-Salatiga, Central Java, Indonesia. The beetroots were cut into pieces, dried, and ground into powder. The beetroot extract was prepared using the maceration method with 96% ethanol for 7 days at room temperature at the Integrated Biomedicine Laboratory, Faculty of Medicine, Sultan Agung Islamic University, Indonesia. Maceration was filtered and then evaporated at 40°C using a rotary evaporator. Evaporation was continued until a thick beetroot was extracted.

Preparation of beetroot extract nanoparticles

Nanoparticles of beetroot extract (NBE) were prepared using the ionic gelation method. The materials used were beetroot extract paste, chitosan, and NaTPP. Na-TPP triggered ionic gelation of beetroot and chitosan. Chitosan and Na-TPP solution were mixed at a ratio of 4:1 (80 mg: 20 mg) in an acetic acid solution (1% w/v) for an hour. Nanoparticle characterization was carried out using a Particle Size Analyzer (PSA) to ensure that the particle size is below 1000 micro and to assess the polydispersity index of the preparation.

Animal experiments

A total of 30 BALB/c male mice aged 2-3 months with a body weight of 25-35 grams were collected from LPPT Gajahmada University and were considered for this study. The experimental mice were divided into 6 groups including 1) H: Baseline (healthy mice), 2) NC: Negative control (inoculated with *P. berghei*), 3) PC: Positive control (inoculated with *P. berghei* + artemisinin (ART) 0.036 mg/g), 4) T1: Treatment 1 (inoculated with *P. berghei* + ART 0.036 mg/g+ NBE 50 mg/kg), 5) T2: Treatment 2 (inoculated with *P. berghei* + ART 0.036 mg/g+ NBE 100 mg/kg), and 6) T3: Treatment 3 (inoculated with *P. berghei* + ART 0.036 mg/g+ NBE 200 mg/kg).

Mice were acclimatized for 7 days before the experiment, then 1 ml of donor mice blood containing 10^7 parasites was inoculated 0,2 ml intraperitoneal into the control and treatment group. The parasitemia index (PI) was calculated on the third day post-inoculation. If the PI was more than 1%, treatment with artemisinin and NBE was administered to positive control and treatment groups (T1, T2, and T3). The administration of artemisinin used Artemisinin 98% (Sigma Aldrich) diluted in saline and given 6 hours before administration of NBE. On the 5th day after the treatment, the experimental animals were terminated for blood specimen collection.

Sample collection

To calculate the PI, the mice blood specimens were used Blood specimen was collected from an orbital vein of mice and used to analyze ICAM-1. The blood samples were collected in a non-heparinized plane tube and were processed into serum through centrifugation at 3000 rpm for 15 minutes. All animals were anesthetized with 0,5 ml ketamin then cervical dislocation was performed.

Calculation of parasitemia index

The blood was dropped on a glass slide spread it smoothly and the sample was dried to form a thin film. Fix the thin film with methanol and dried. Stained the thin film with Giemsa for 20-30 minutes. The slide was washed with ample water and dried in a tilted position. The slide was observed under a microscope at 1000x magnification with immersion oil. The PI was calculated according to the formula as follows:

$$PI\ (\%) = \frac{Infected\ erythrocytes}{1000\ erythrocytes}\ x\ 100\%$$

Measurement of serum ICAM-1 levels

The serum ICAM-1 level was measured using the ELISA method and ELISA mouse ICAM-1/CD54 reagent (Elabscience Cat No. E-EL-M3037). Briefly, 100 µl blank, standard, and serum were added to the microplate, mixed, and then incubated for 90 minutes at 37°C. The liquid was discarded, and 100 µl of the working solution was added to each well. The plate was covered and incubated for an hour at 37°C. Discarded the liquid and washed with a solution of all wells, repeating these steps thrice. Briefly, 100 µl of HRP solution was added to each and then incubated at 37°C for 30 minutes. All liquid was discarded, and each well was washed with washing buffer, repeated this step 5 times. Then, 90 µl of the substrate was added to each well and incubated for 15 minutes at 37°C, and then the plate was kept in the dark. Finally, 50 µl of stop solution was added to each plate well. The optical density (OD) was determined using a microplate reader at a wavelength of 450 nm.

Statistical analysis

The data were statistically analyzed. Kruskal Wallis and Mann Whitney tests were used to investigate the effect of beetroot extract nanoparticles on the PI and ICAM-1 levels. The Student T-test was used to compare the groups. The statistical analysis was presented using median, percentile, and p-value, in which a p-value <0.05 is considered statistically significant. Statistical analysis was performed using IBM SPSS statistic 22.0.

RESULTS

Effect of beetroot extract on the parasitemia index

Table 1 shows that the PI in mice infected with *P. berghei* was 36%. Administration of artemisinin in the positive control group had a lower PI than the negative control, namely 16.2%. Supplementation with various doses of beetroot extract nanoparticles together with artemisinin in the group inoculated with *P. berghei* resulted in a lower PI (T1-15.6%, T2-13.1%, and T3-12.5%) compared to the NC group.

The data showed that the PI was significantly higher in the NC group. In contrast, the PI in treatment groups T2 and T3 were significantly lower compared to the positive and negative control groups ($p<0.05$). These results suggest that beetroot extract nanoparticle doses (100 mg/kg/day and 200 mg/kg/day) act as an anti-malarial agent by reducing parasitemia in mice infected with malaria and treated with artemisinin (Figure 1).

Table 1. The effect of beetroot extract nanoparticles in PI of mice inoculated with *P. berghei.*

	Parasitemia index (%)	P value
B	0	
NC	36 (30.9-41.1)*	
PC	16.2 (15.6-16.7) #	0.002*
T1 (ART+NBE 50 mg)	15.6 (13.4-19.4) #	
T2 (ART+NBE 100 mg)	13.1 (11.9-16.3) #	
T3 (ART+NBE 200 mg)	12.5 (9.7-15.7) #	

B: Baseline, NC= negative control (inoculated with *P. berghei*), PC= positive control (inoculated with *P. berghei* + artemisinin 0.036 mg/g), T1= treatment 1(inoculated with *P. berghei* + artemisinin 0.036 mg/g+ nanoparticle of beetroot extract 50 mg/kg), T2= treatment 2 (inoculated with *P. berghei* + artemisinin 0.036 mg/g+ nanoparticle of beetroot extract 100 mg/kg), T3= treatment 3(inoculated with *P. berghei* + artemisinin 0.036 mg/g+ nanoparticle of beetroot extract 200 mg/kg). ART=artemisinin, NBE=nanoparticle of beetroot extract. Data is presented as median (n=5 for each group), * $p<0.05$ compared to the baseline, and # $p<0.05$ compared to the negative control.

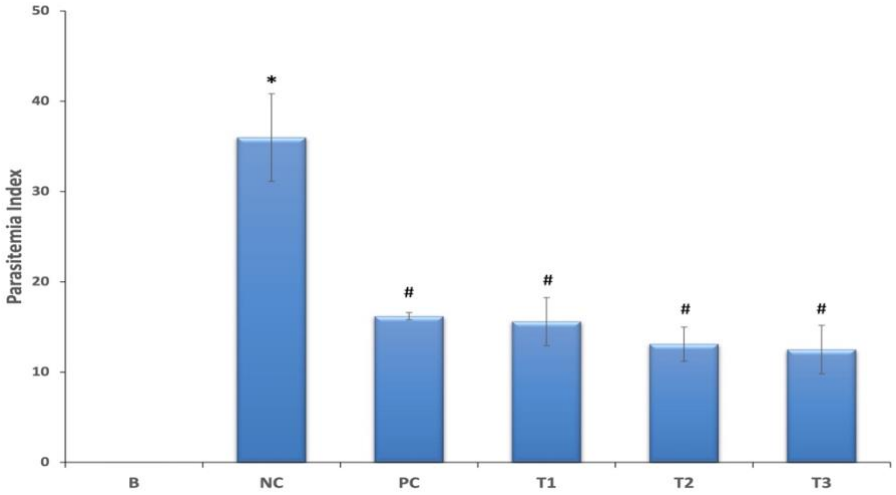

Figure 1. Effect of nanoparticle of beetroot extract in the parasitemia index on mice inoculated with *Plasmodium berghei* ANKA and treated with artemisinin. n=5 at five days interval after treatment. Figures indicate the median value, B: Baseline, NC= negative control (inoculated with *P. berghei*), PC= positive control (inoculated with *P. berghei* + artemisinin 0.036 mg/g), T1= treatment 1(inoculated with *P. berghei* + artemisinin 0.036 mg/g+ nanoparticle of beetroot extract 50 mg/kg), T2= treatment 2 (inoculated with *P. berghei* + artemisinin 0.036 mg/g+ nanoparticle of beetroot extract 100 mg/kg), T3= treatment 3(inoculated with *P. berghei* + artemisinin 0.036 mg/g+ nanoparticle of beetroot extract 200 mg/kg). Data is presented as median (n=5 for each group), * p<0.05 compared to the baseline, and # p<0.05 compared to the negative control.

Effect of beetroot extract nanoparticle on the level of ICAM-1 in infected mice

Table 2 shows that ICAM-1 level increased in *P. berghei*-infected mice for 7 days, where the ICAM-1 level was 319.17 ng/ml. Administration of artemisinin to the positive control group resulted in the reduction of ICAM-1 level compared to the negative control, where the ICAM-1 level was 241.93 ng/ml. Supplementation with beetroot extract nanoparticles together with artemisinin has resulted in the reduction of ICAM-1 level compared to the NC mice, where the ICAM-1 levels were 193.65 ng/ml (T1), 149.65 ng/ml (T2), and 151.24 ng/ml (T3), respectively.

The ICAM-1 levels in the negative and positive control groups were significantly higher than the baseline group (p=0.009), while treatment groups 2 and 3 were significantly lower than the negative and positive control groups (p=0.009). ICAM-1 levels in the treatment groups 2 and 3 showed no significant difference to the baseline group. These findings suggest that beetroot extract nanoparticle doses 100 mg/kg/day and 200 mg/kg/day can act as an anti-inflammatory agent by reducing ICAM-1 level in mice infected with malaria and treated with artemisinin (Figure 2).

Table 2. Effect of beetroot extract nanoparticle in ICAM-1 level in *P. berghei*-inoculated mice

Treatment	ICAM-1 (ng/ml) level	P value
B	200.21 (103.65-222.96)	0.001
NC	319.17 (254-334.34)*	
PC	241.93 (224.68-262.62)#	
T1 (ART+NBE 50 mg)	193.65 (135.72-256.41) #	
T2 (ART+NBE 100 mg)	145.72(121.24-182.62)#	
T3 (ART+NBE 200 mg)	151.24 (121.59-165.38)#	

B: Baseline, NC= negative control (inoculated with *P. berghei*), PC= positive control (inoculated with *P. berghei* + artemisinin 0.036 mg/g), T1= treatment 1(inoculated with *P. berghei* + artemisinin 0.036 mg/g+ nanoparticle of beetroot extract 50 mg/kg), T2= treatment 2 (inoculated with *P. berghei* + artemisinin 0.036 mg/g+ nanoparticle of beetroot extract 100 mg/kg), T3= treatment 3(inoculated with *P. berghei* + artemisinin 0.036 mg/g+ nanoparticle of beetroot extract 200 mg/kg). ART=artemisinin, NBE=nanoparticle of beetroot extract. Data is presented as median (n=5 for each group), when * p<0.05 compared to the baseline, and # p<0.05 compared to the negative control.

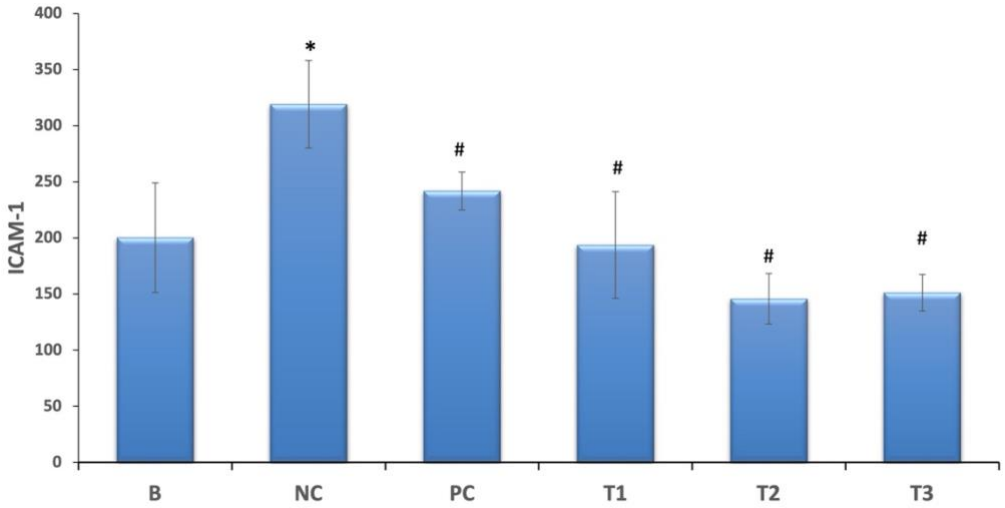

Figure 2. Effect of Beetroot Extract Nanoparticle in the ICAM-1 serum level on mice inoculated with *Plasmodium berghei* ANKA and treated with artemisinin. n=5 at five days interval after treatment. Figures indicate the median value, B=Baseline (healthy control), NC= negative control (inoculated with *P. berghei*), PC= positive control (inoculated with *P. berghei* + artemisinin 0.036 mg/g), T1= treatment 1(inoculated with *P. berghei* + artemisinin 0.036 mg/g+ nanoparticle of beetroot extract 50 mg/kg), T2= treatment 2 (inoculated with *P. berghei* + artemisinin 0.036 mg/g+ nanoparticle of beetroot extract 100 mg/kg), T3= treatment 3 (inoculated with *P. berghei* + artemisinin 0.036 mg/g+ nanoparticle of beetroot extract 200 mg/kg). Values with superscripts are statistically significant when * $p<0.05$ compared to the baseline, and # $p<0.05$ compared to the negative control.

DISCUSSION

This research shows that *P. berghei* ANKA inoculation can increase the PI and can cause malaria infection in experimental animals. However, supplementation of beetroot extract nanoparticles significantly modulates the immune system by reducing ICAM-1 levels and PI confirmed in *P. berghei* inducing malaria in mice.

Hemozoin is an energy source for *plasmodium* produced due to heme polymerization in the *plasmodium* food vacuole. Betalain in beetroot inhibits the polymerization of heme into hemozoin, and free heme can be toxic to *plasmodium* [16] (reference required). The quaternary nitrogen content in beetroot also plays a role in inhibiting the number of parasitemia by regulating parasite cell membrane biogenesis. Serine and choline in the patient's serum encounter intracellular movement to the *plasmodium*. Serine moves to the parasite's cytoplasm, in which it converts into ethanolamine. Phosphorylation of ethanolamine becomes phosphoethanolamine, which is then converted together with choline to form phosphocholine, a precursor of phosphodylcholine. The *Pfpmt* enzyme in *Plasmodium* induces methylation of phosphocholine to become phosphodylcholine, which plays a role in *Plasmodium* membrane biogenesis [16]. Disruption of heme polymerization into hemozoin and parasite membrane biogenesis leads to damage and causes death to *Plasmodium*, which is the consequence of the reduction in the PI with beetroot extract nanoparticle supplementation [16–18].

Malaria infection shows an increase of ICAM-1 level on the 7th day with *P. berghei* inoculation in the negative control group. The administration of artemisinin to the positive control showed that ICAM-1 levels were lower than in the negative control, while supplementation with beetroot extract nanoparticles, while artemisinin in the treatment group showed that ICAM-1 levels decreased with the increasing doses of beetroot extract nanoparticles. This proves that the supplementation of beetroot extract nanoparticles together with artemisinin inhibits the increase of ICAM-1 levels in malaria-infected mice.

ICAM-1 is a glycoprotein that is expressed on the surface of blood vessel endothelial cells, and it regulates leukocyte recruitment. ICAM-1 plays a role in the adhesion of infected erythrocytes to the endothelium and the occlusion and ischemia of blood vessels, which causes various types of organ failure in severe malaria[19]. *P. berghei* inoculation causes increased parasitemia in mice, this also increases infected erythrocytes (P-RBC) and erythrocyte lysis. As a result, there is an increase in heme degradation and the formation of hemozoin, which is accompanied by the release of free radicals. Increased production of free radicals l triggers oxidative stress and tissue damage, this then leads to the stimulation of the innate immune response and excessive release of pro-inflammatory cytokines such as TNF-α and IL-1 [4]. Hemozoin produced by *plasmodium* also activates NF-κB (Nuclear factor kappa B), leading to the pathogenesis of severe malaria. Moreover, activation of NF-κB stimulates the activation of matrix metalloproteinase 9 ((MMP-9), and induces the production of pro-inflammatory cytokines such as TNF-α and IL-1β. The production of pro-inflammatory cytokines, such as TNF-α and IL-1, is the output of NF-κB activation under malaria-infected conditions. The production of proinflammatory cytokines stimulates the expression of ICAM-1, which is released in the blood during the form of sICAM-1 [6,20,21].

Beetroot extract contains active ingredients including betalain, betaxanthin, betanin, and indicaxanthin which have anti-inflammatory properties. Betalain and indicaxanthin inhibit the binding of NF-κB and DNA; thereby they are downregulating the formation of pro-inflammatory cytokines. Furthermore, betalain also plays a role in modulating Nrf2-Keap1 and NF-κB-IKB binding, which leads to increasing free Keap1 in the cytosol. Keap-1 will be bonded to ikB Kinase (IKK) and inhibit IKB phosphorylase by IKK. This process inhibits NF-κB-IKB binding that triggers NF-κB translocation and activation [22–24].

The inhibition of NF-κB activation causes a decrease in the production of pro-inflammatory cytokines such as TNF-α and IL-1β, which reduce the expression of ICAM-1 in the blood vessel endothelium and the release of ICAM-1 in the blood circulation. Betanin also plays a role in stimulating the production of anti-inflammatory cytokines by macrophages, which is IL-10 inhibits the activation of macrophages, Th1 cells, and inhibits the activation of NF-κB, resulting in reduced production of pro-inflammatory cytokines, which triggers a decrease in ICAM-1 expression [25,26].

Beetroot-extracting nanoparticles increase the effectiveness of active substances in the body. This ability of beetroot extract to inhibit the increase in the PI, and control the excessive increase in pro-inflammatory cytokines produced due to the immune response to malaria infection. The beetroot activity stimulates an increase in anti-inflammatory cytokines, which suppress ICAM-1 expression on blood vessel endothelium, leading to the release of ICAM-1 levels in the blood. Lower ICAM-1 levels due to beetroot extract nanoparticle supplementation prevent PRBC sequestration and blood vessel blocking, thus preventing organ failure caused by severe malaria infection [26].

Although several studies have been conducted on beetroot extract in different infectious diseases. However, this is the first study to integrate the application of beetroot extract nanoparticles with artemisinin in malaria infection. The results have demonstrated potential as an adjuvant therapy against malaria. However, further research is needed to assess the potential effect of beetroot extract nanoparticles in preventing severe malaria; it also needs to explore its role in resolving post-disease complications and screening cytotoxicity to ensure the safety of drug formulation in malaria-infected patients.

CONCLUSIONS

This study explores the potential of beetroot-extracting nanoparticles against malaria disease. The PI and ICAM-1 levels increased in the group of infected mice. However, the supplementation of beetroot extract nanoparticles at doses 100 mg/kg/day and 200 mg/kg/day in the malaria-infected group treated with artemisinin showed a reduction in both the PI and ICAM-1 levels. These outputs beetroot extract component was active malaria-infected mice. The active compounds, especially betanin and flavonoid in beetroot extract nanoparticles, were involved in inhibiting the PI and ICAM-1 levels. The overall findings suggest the potential of beetroot extract nanoparticles could be used as a potential adjuvant against malaria and as an anti-inflammatory and anti-malarial agent.

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AUTHOR CONTRIBUTIONS

FH conceptualized, performed the experiment, analyzed data, and interpreted the results. LS and KD contributed suggestions, checked on its interpretation data analysis, and reviewed the manuscript. All authors approved the final version of the manuscript.

CONFLICTS OF INTEREST

There is no conflict of interest among the authors.

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