

In vivo antimalarial activity of stem bark extracts of *Plumeria alba* against *Plasmodium berghei* in imprinting control region mice

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Background: The need for new antimalarial agents with a transcriptional mode of action but fewer side effects compared with artemisinin-based combination therapy for malaria has been the preoccupation of scientists in areas where malaria is a menace. Stem bark extracts of *Plumeria alba*, used traditionally for the treatment of malaria in Ghana, were investigated to evaluate their prophylactic and curative antimalarial properties.

Methods: The antimalarial properties of *P. alba* were probed using aqueous (30–300 mg/kg) and dichloromethane/methanol (30–300 mg/kg) extracts of the plant in imprinting control region mice infected with *Plasmodium berghei*. For the curative test, the extracts were administered to the infected mice 4 days post-infection. In the prophylactic test, the animals were pre-treated with the extracts for 3 days before challenging them with *P. berghei* infected erythrocytes.

Results: The aqueous extract exerted significant ($P < 0.05$ – 0.001) effects on *P. berghei* infection, similar to artemether and lumefantrine curatively and sulfadoxine/pyrimethamine prophylactically. However, the dichloromethane/methanol extract reduced the parasitemia curatively ($P < 0.05$ – 0.01) but not prophylactically.

Conclusion: This study provides evidence to support the antimalarial properties of stem bark extract of *P. alba* in mice.

Keywords: malaria, parasitemia, artemether, lumefantrine, sulfadoxine/pyrimethamine, Ghana

Introduction

Malaria is a major global public health problem and is the leading poverty-associated disease, directly undermining the achievements of four millennium development goals: the eradication of extreme poverty, reduction of child mortality rates, improvement of maternal health, and combating of HIV/AIDS and other diseases.¹ The alarming rate at which malaria parasites develop resistance to most of the available and affordable antimalarial drugs is a major concern that urgently requires the development of newer and more effective alternatives.²

In the present study, *Plumeria alba*, commonly known as “white frangipani,” from the family Apocynaceae, was studied for its antimalarial activity. Phytochemically, the leaves of *P. alba* contain terpenoids, flavonoids, alkaloids, glycosides, phytosteroids, tannins, and carbohydrates.³ Traditionally, the latex from the leaves is used to manage ulcers and herpes. The seeds possess hemostatic properties and the bark is applied as plaster over hard tumors. In addition, the leaves and bark are employed as diuretic, purgative, cardiotoxic, and hypotensive agents.⁴

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Although *P. alba* stem bark is commonly used traditionally for the treatment of malaria in Ghana, there is little scientific data to support its use for this purpose. Therefore, the study reported here examined the in vivo curative and prophylactic effects of dichloromethane/methanol and aqueous stem bark extracts of *P. alba* against *Plasmodium berghei* in imprinting control region (ICR) mice, envisaging that the results could be extrapolated to cover human malaria parasites.

Materials and methods

Chemicals and test agents

The methanol and dichloromethane used for the extractions were purchased from Sigma-Aldrich (St Louis, MO, USA), the artemether and lumefantrine (A-L) were obtained from Ajanta Pharma Ltd (Mumbai, India), and the sulfadoxine/pyrimethamine (SP) was obtained from Maxheal Labs Pvt Ltd (Sachin, India).

Collection and identification of plant material

Stem bark of *P. alba* was obtained from a local herbalist (Alahji Sadik Abubakr of AA Sadik Herbal Centre, Cape Coast, Ghana). The bark was identified and authenticated by a botanist at the University of Cape Coast Herbarium. A voucher specimen with reference number Bio/BMS/161 was prepared and deposited at the Herbarium for reference.

Preparation of extracts

The stem bark of *P. alba* was washed thoroughly with tap water and sundried. The dry bark was chopped into pieces and milled into coarse powder with a hammer mill (Schutte Buffalo Hammermill, New York, NY, USA). In preparing the aqueous extract of *P. alba*, 200 g of the stem bark powder was mixed with 2 L of distilled water. The mixture was maintained at 80°C (in a round-bottomed flask fitted with a reflux condenser) in a thermostatically controlled water bath for 48 hours and then filtered. The filtrate was freeze-dried with a Hull Corp 140 Square Foot Freeze Dryer/Lyophilizer (model 140FS275C; Warminster, PA, USA) into powder (yield 5.2%) and stored at a temperature of 4°C in a refrigerator. The powder was reconstituted in normal saline to the desired concentration and labeled “aqueous extract of *P. alba*” (referred to hereafter as “aqueous extract”) for dosing.

Similarly, 200 g of the stem bark powder was soaked in 2 L of solvent (dichloromethane/methanol [1:1]) at room temperature for 48 hours then filtered. The filtrate obtained was freeze-dried into powder (yield 3.4%). Quantities of this

powder were reconstituted in normal saline at the desired concentrations to be used in this study and labeled “dichloromethane/methanol of *P. alba*” (D/M) extract.

Screening for secondary metabolites

The aqueous and D/M extracts were screened to ascertain the presence of phytochemicals using standard procedures described elsewhere.⁵⁻⁸

Animals and husbandry

Eight-week-old male ICR mice (25–30 g) purchased from the Centre for Scientific Research into Plant Medicine, Mampong-Akwapim, Ghana, were maintained in the Animal House of the Department of Biomedical and Forensic Sciences, University of Cape Coast, Cape Coast, Ghana. The animals were housed in stainless steel cages (34 × 47 × 18 cm) with soft wood shavings as bedding, under ambient laboratory conditions. They were fed on a normal commercial pellet diet (Agricare Ltd, Kumasi, Ghana) and had free access to water. All procedures and techniques used in these studies were in accordance with the National Institute of Health for the Care and Use of Laboratory Animals.⁹ Protocols for the study were approved by the departmental ethics committee (BMS/13/10).

Source of rodent parasite and inoculation of *P. berghei* NK65

The rodent parasite was sourced from Noguchi Memorial Institute for Medical Research, University of Ghana, Legon, Ghana and maintained alive in mice by continuous intraperitoneal passage every 5 days.

On the day of the experiment, a total inoculum concentration of 9.6×10^7 of *P. berghei* parasitized erythrocytes per mL was prepared. This was carried out by determining parasite density of the *P. berghei* in the blood of infected mice. The infected blood was first washed with ethylenediaminetetraacetic acid (EDTA)-phosphate-buffered saline (PBS) and then washed twice with only PBS. It was then diluted appropriately (by a factor of 96) with PBS. Following this, each mouse was inoculated on the first day, Day 0, with 0.2 mL of infected blood containing 1×10^6 *P. berghei* parasitized red blood cells.

Effect of aqueous and D/M extracts of *P. alba* on established *P. berghei* infection

To assess the curative potential of the aqueous and D/M extracts on established *P. berghei* infection, 48 male mice were each inoculated with 1×10^6 *P. berghei* on Day 0. They were then randomly assigned to eight groups (n = 6).¹⁰

The mice were monitored to ensure establishment of the infection. After 72 hours, each group was treated orally with 30, 100, or 300 mg/kg/day of aqueous extract (Groups 1–3); 30, 100, or 300 mg/kg/day of D/M extract (Groups 4–6); 4 mg/kg/day of A-L (standard drug; Group 7); and 10 mL/kg/day normal saline daily (control; Group 8) for 5 days. These doses were chosen based on preliminary study in our laboratory. To determine the effectiveness of the extracts, blood was drawn from the tail to determine the parasitemia using the formula: percentage parasitemia = infected red blood cells in 100 fields \times 100, divided by total red blood cells (2,000) in the same fields.

A minimum of 100 high-power fields were examined before a thin film was declared negative. Each slide was read independently by two experienced microscopists.

The mean survival time of the mice in each treatment group was determined over a period of 30 days.

Histopathological assessment

On Day 12, two animals from each group were randomly selected and sacrificed. Their livers were harvested, fixed in 4% phosphate-buffered paraformaldehyde, and embedded in paraffin. Sections were stained with hematoxylin and eosin, and fixed on glass slides for microscopic examination by an anatomist at the Department of Biomedical and Forensic Sciences, University of Cape Coast.

Prophylactic activity of aqueous and D/M extracts of *P. alba* on *P. berghei* infection

The method described by Peters¹¹ was used to evaluate the prophylactic activity of the extracts. Briefly, 48 male mice were randomly assigned to eight groups (n = 6) and each group was pre-treated orally with 30, 100, or 300 mg/kg/day of the aqueous extract (Groups 1–3); 30, 100, or 300 mg/kg/day of the D/M extract (Groups 4–6); 1.2 mg/kg/day SP (the reference drug; Group 7); or 10 mg/kg/day normal saline (Group 8). The treatment was continued for 3 consecutive days. On the fourth day, all mice were infected with 1×10^6 *P. berghei* and, 72 hours later, blood drawn from the tail of the mice was used to prepare thick films. The parasite density and percentage chemosuppression for all the treatment groups were determined.

Statistical analysis

GraphPad Prism for Windows (v 4.03; GraphPad Software, San Diego, CA, USA) was used for all statistical analyses and *P* values < 0.05 were considered statistically significant. All data were expressed as mean \pm standard error of the mean

(duplicate measurement). The time–course curves were subjected to two-way (treatment \times time) repeated-measures analysis of variance (ANOVA) with Bonferroni's post hoc test. The column graphs were subjected to one-way ANOVA with Tukey's post hoc test.

Results

Phytochemical screening of the extracts revealed the presence of glycosides and terpenoids for both extracts. Additionally, the D/M extract contained coumarins while the aqueous extract contained anthracenes (Table 1).

The aqueous and D/M extracts, as well as the standard antimalarial drug A-L, reduced the parasitemia significantly from the first day of treatment to the final day. All the treatments provided relatively increased the survival time of the mice compared with the control (Table 2). The aqueous extract significantly ($P < 0.0001$) reduced the level of parasitemia from Day 4 post-infection and achieved the highest effect on Day 8 (Figure 1A). The percentage change (% Δ) of parasitemia reduction caused by the 30 mg/kg aqueous extract was 16.25%, 22.2%, 22.5%, and 8.4% on Days 5, 6, 7, and 8 post-infection, respectively. The 100 mg/kg aqueous extract treatment also produced a % Δ of parasitemia reduction of 18.9%, 27.3%, 6.2%, and 23.4% on Days 5, 6, 7, and 8 post-infection. Similarly, the % Δ of parasitemia reduction by the 300 mg/kg aqueous extract was 32.2%, 33.3%, 16.4%, and 16.4% on Days 5, 6, 7, and 8 post-inoculums, respectively (Figure 1A).

The D/M extract significantly ($P < 0.0001$) reduced the level of parasitemia from Day 4 post-infection and achieved the highest effect on Day 8. The % Δ of parasitemia reduction by the 30 mg/kg D/M extract was 23.2%, 4.7%, 23.4%, and 20% on Day 5, 6, 7, and 8 post-inoculums, respectively (Figure 1B). The % Δ of parasitemia reduction by the 100 mg/kg D/M extract was 38.1%, 12.4%, 24.3%, and 29.1% on Days 5, 6, 7, and 8 post-inoculums, respectively. The 300 mg/kg D/M extract produced a % Δ of parasitemia reduction of 34.3%, 30.5%, 31.4%, and 24.4% respectively

Table 1 Phytochemical screening of *Plumeria alba* stem bark

Secondary metabolite tested	Aqueous extract	D/M extract
Alkaloids	–	–
Glycosides	+	+
Anthracenes	+	–
Terpenoids	+	+
Flavonoids	–	–
Coumarins	–	+

Notes: + = present; – = absent.

Table 2 Summary of the effects of aqueous extract of *Plumeria alba* (aqueous), dichloromethane/methanol extract of *P. alba* (D/M), and artemether and lumefantrine (A-L) on established *Plasmodium berghei* infection in mice

Drug	% parasitemia	% chemosuppression	Survival days
Control	35.5 ± 3.1	–	12 ± 0.1
Aqueous 30 mg/kg	14.6 ± 1.8***	58.9	12 ± 0.4
Aqueous 100 mg/kg	12.2 ± 1.6***	65.6	13 ± 0.4
Aqueous 300 mg/kg	11.2 ± 1.1***	68.5	19 ± 0.3
D/M 30 mg/kg	8.7 ± 1.7***	75.6	16 ± 0.3
D/M 100 mg/kg	7.8 ± 2.5***	78.7	18 ± 0.5
D/M 300 mg/kg	7.1 ± 1.8***	80.1	25 ± 0.6
A-L 4 mg/kg	0.14 ± 0.1***	96.2	28 ± 0.2

Notes: Values are expressed as mean ± standard error of the mean. ***P ≤ 0.001.

on Days 5, 6, 7, and 8 post-inoculums, respectively. The standard antimalarial drug A-L (4 mg/kg) produced a % Δ of parasitemia reduction of 40.4%, 42.9%, 76.9%, and 94% on Days 5, 6, 7, and 8 post-inoculums, respectively (Figure 1B).

A-L produced the highest % chemosuppression of 99.6% on Day 8, which was 1.5 times the highest % chemosuppression produced by the aqueous extract (300 mg/kg) and 1.2 times the highest % chemosuppression produced by the D/M extract (Table 2).

Figure 2A–H represent the histopathological assessment of liver in the treated groups of animals. Numerous Kupffer cells were observed in all tissue sections, except

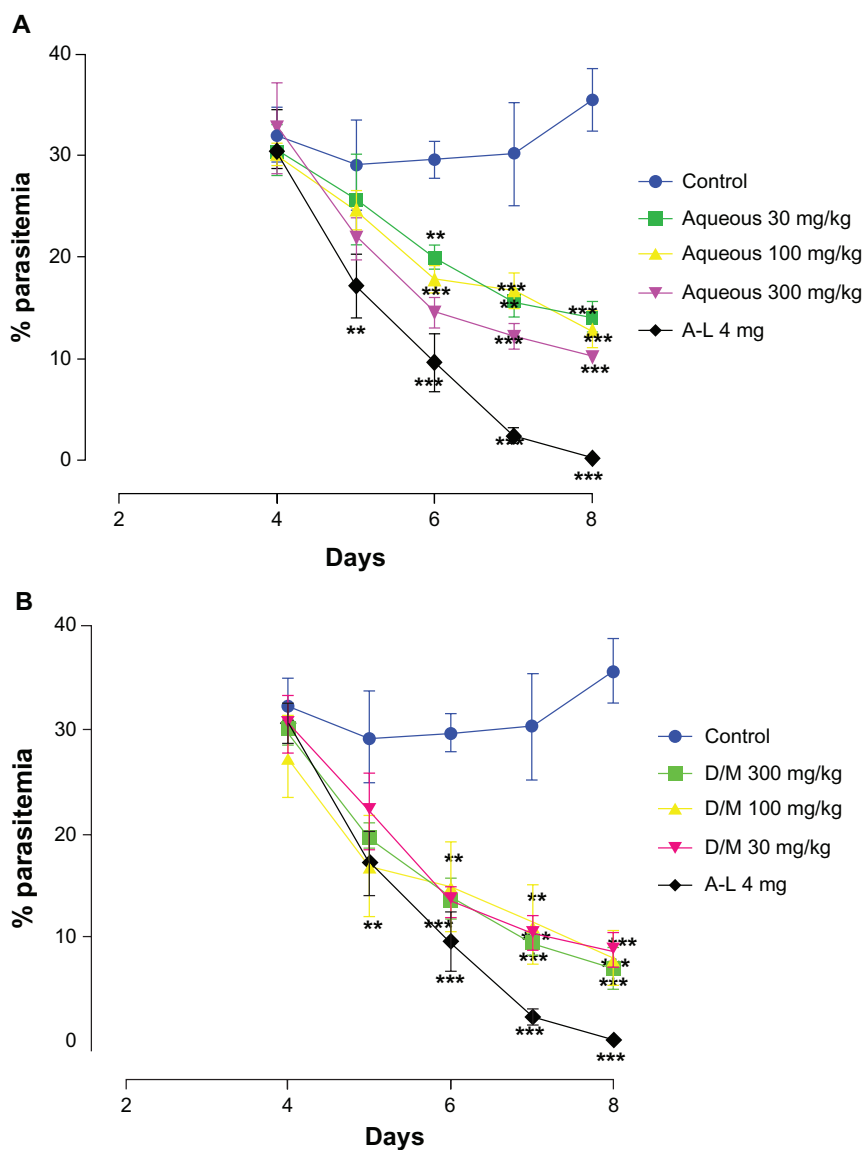


Figure 1 Effect of (A) aqueous extract of *Plumeria alba* (aqueous) and artemether and lumefantrine (A-L) and (B) dichloromethane/methanol extract of *P. alba* (D/M) and A-L on the time-course curve of *Plasmodium berghei* infection in mice.

Notes: Data are presented as mean ± standard error of the mean. **P < 0.01 and ***P < 0.001 compared with the vehicle-treated group (two-way analysis of variance followed by Bonferroni's post hoc test).

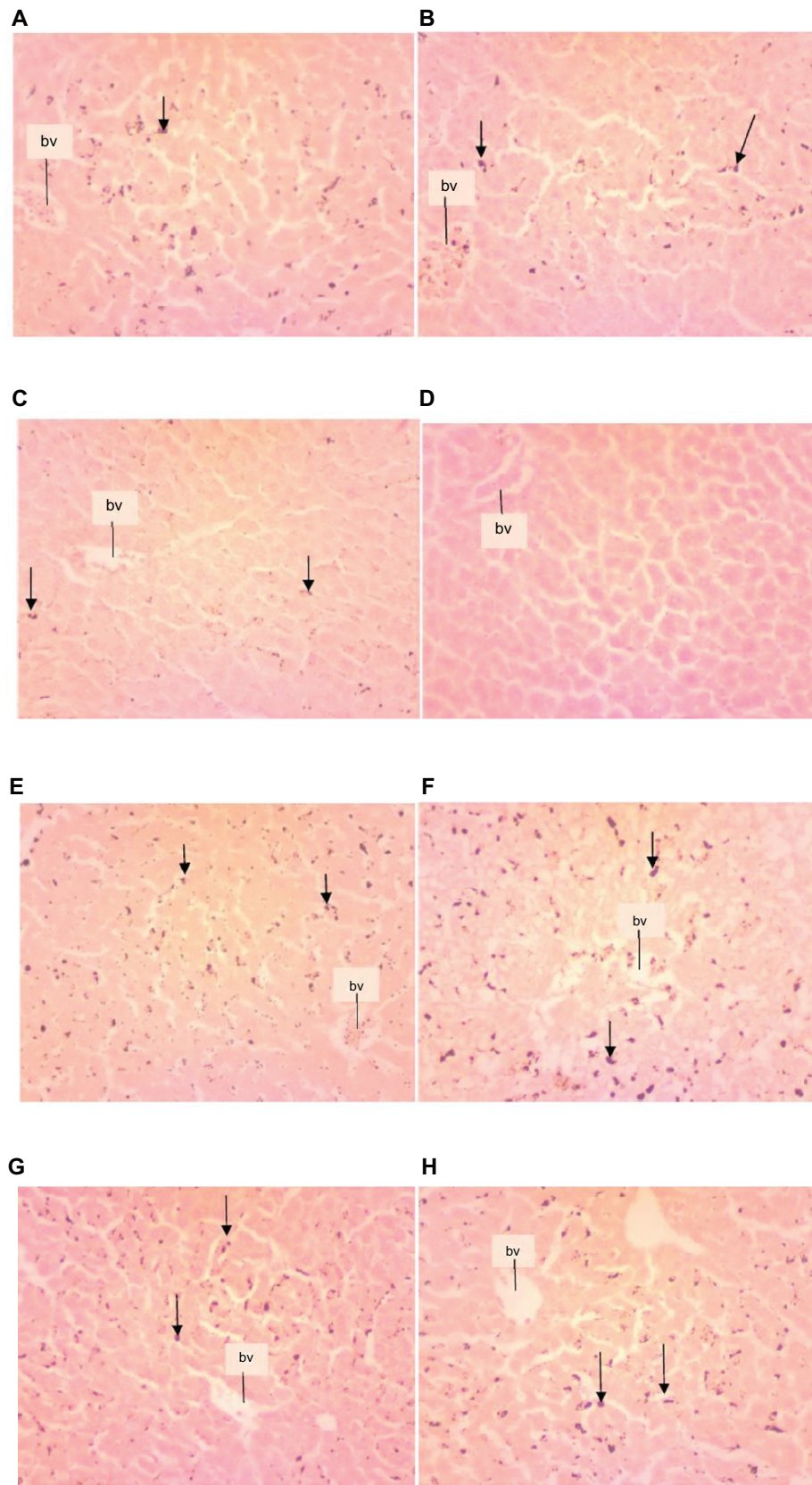


Figure 2 Histopathological assessment of liver cells of mice infected with *Plasmodium berghei*.

Notes: (A) Control (normal saline); (B) dichloromethane/methanol extract of *Plumeria alba* (D/M) 30 mg/kg; (C) D/M 100 mg/kg; (D) D/M 300 mg/kg; (E) aqueous extract of *P. alba* (aqueous) 30 mg/kg; (F) aqueous 100 mg/kg; (G) aqueous 300 mg/kg; (H) artemether and lumefantrine 4 mg/kg. Arrows signify Kupffer cells.

Abbreviation: bv, lumen of blood vessel.

animals treated with 300 mg/kg D/M extract (Figure 2D). The numerous Kupffer cells observed could be attributed to inflammatory reactions in the liver as result of parasite protein sequestration.

The aqueous extract exhibited significant ($P < 0.05$) prophylactic activity against *P. berghei* in vivo at all the three doses tested (Figure 3A) seen as reduction in parasite count compared to the vehicle-treated group. The % chemosuppressive effect seen at the highest dose employed was 49.4%, which was 1.1 times lower than that of the standard drug SP (% chemosuppression of 58.2%) (Table 3). However, the D/M extract did not show significant prophylactic activity against

P. berghei in vivo (Figure 3B). The maximum % chemosuppressive effect produced by D/M extract was 36.4%, 1.6 times lower than that of SP (Table 3).

Discussion

Rodent models of malaria are recommended as a convenient model for preclinical studies of drugs for use in the treatment of human Plasmodial infection. There is ample evidence of the validation of common conventional antimalarial drugs using these models.¹⁰

The aqueous extract, D/M extract, and A-L exerted curative activities against *P. berghei* infection in the mice, although the D/M extract was not effective prophylactically. The extracts increased the survival time of the mice when compared with survival time in the vehicle-treated groups.

The aqueous and D/M extracts suppressed the growth of *P. berghei* malaria in the curative assay, with highest malaria suppression at 300 mg/kg. The percentage decrease in parasitemia was comparable to 4 mg/kg A-L, though A-L had the highest chemosuppression. This suggests that *P. alba* stem bark extract can suppress parasite growth if given orally for curative purposes. The oral treatment with *P. alba* on Day 8 revealed that the aqueous and D/M extracts had reduced the level of parasitemia to 16.4% and 20.0%, respectively. Again, the histological assessment of mice hepatocytes revealed relatively low presence of Kupffer cells in tissue treated with 300 mg/kg D/M extract as compared with that treated with 4 mg/kg A-L.

The presence of phytochemicals such as terpenoids, coumarins, glucosides, and anthracenes observed during qualitative screening for plant secondary metabolites suggests that the extracts may exert some mechanisms that counter the pathological processes of *P. berghei* infection. These metabolites, with profound antioxidant properties, among other mechanisms,^{12,13} may exert their antimalarial effect by decreasing nitric oxide production in Kupffer cells, resulting in killing the parasites.¹⁴ In addition, secondary metabolites such as anthracenes and glycosides have been shown to possess direct anti-plasmodial effects. It is likely that the aqueous and D/M extracts could contain such anthracenes and glycosides, accounting for their anti-plasmodial effect.^{13,15} The relative increase in mean survival time of the extract and A-L treatment groups was due to the clearance of the parasites from the blood of these animals.

For the prophylactic treatment, the D/M extract was not effective, as shown by lower percentages of chemosuppression compared with those of aqueous extract and SP.

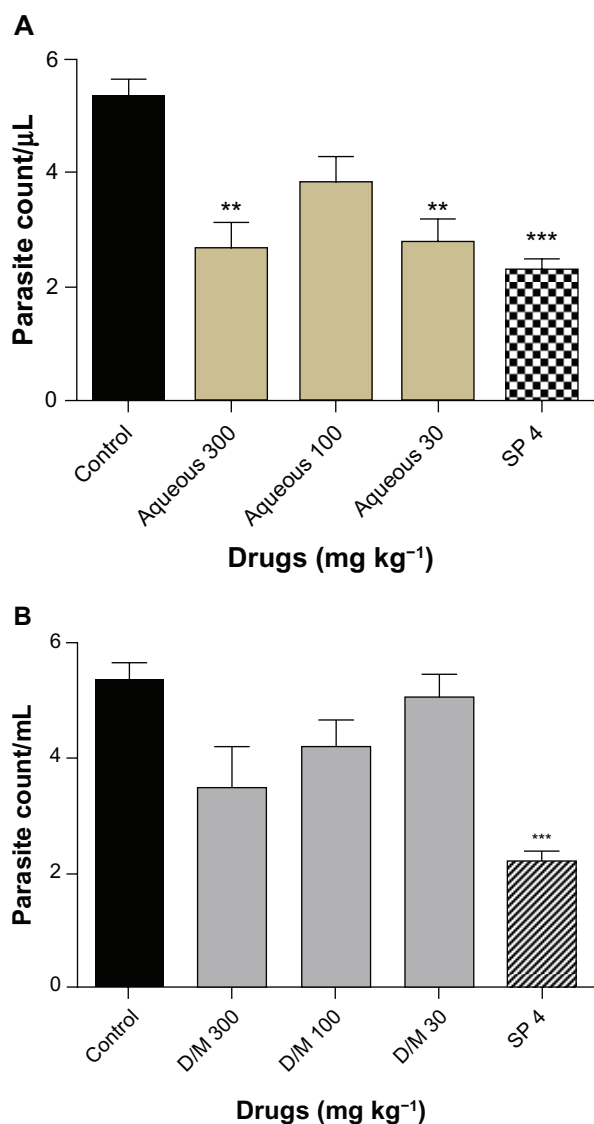


Figure 3 Prophylactic effect of (A) aqueous extract of *Plumeria alba* and sulfadoxine/pyrimethamine (SP) and (B) dichloromethane/methanol extract of *Plumeria alba* (D/M) and SP on residual malaria infection of *Plasmodium berghei* in mice.

Notes: Data are presented as mean \pm standard error of the mean. *** $P < 0.001$, ** $P < 0.01$ compared with vehicle-treated group (one-way analysis of variance followed by Tukey's post hoc test).

Table 3 Summary of the effects of aqueous extract of *Plumeria alba* (aqueous), dichloromethane/methanol extract of *P. alba* (D/M), and sulfadoxine/pyrimethamine (SP) on residual *P. berghei* infection in mice

Parameter	Control (vehicle)	D/M (mg/kg)			Aqueous (mg/kg)			SP (mg/kg)
		30	100	300	30	100	300	4
% chemosuppression	–	7.2	23.6	36.4	50.9	28.3	47.2	58.2

The aqueous extract and SP had similar percentage chemosuppression against *P. berghei*-infected mice as curative agents. The ineffectiveness of D/M extract against *P. berghei* prophylactically may be due to the rapid clearance of the extract or the absence of anthracenes that were present in the aqueous extract.

Conclusion

As far as the authors are aware, the study reported here is the first to have demonstrated that both D/M and aqueous extracts of *P. alba* possess curative antimalarial activities. However, only the aqueous extract possesses prophylactic activity. The efficacy of *P. alba* stem extract can be attributed to the presence of active secondary plant metabolites (coumarins, anthracenes, terpenoids, and glycosides) acting either independently or in concert.

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Disclosure

All authors contributed equally to this work and declare no conflicts of interest in relation to it.

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