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In vivo antiplasmodial activity of the methanol leaf extract of *Piliostigma reticulatum* (Dc.) Hochst (Fabaceae)

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Abstract

Background: *Piliostigma reticulatum* is a plant traditionally used to treat malaria, smallpox, neuralgia, dysentery, diarrhea, and rheumatism in Northern Nigeria. There is no scientific evidence to support the antimalarial activity of this plant. This work aims to investigate the in vivo antiplasmodial activity of the methanol leaf extract of *Piliostigma reticulatum* (MPR) in mice, infected with NK65 chloroquine-sensitive *Plasmodium berghei*. The oral lethal doses and preliminary phytochemical screening of the extract were performed. The therapeutic, suppressive, and prophylactic models were used for the antiplasmodial activity at the doses of 250, 500, and 1000 mg/kg of the MPR extract. Chloroquine and artesunate were used as the positive control drugs, while distilled water was used for the negative control group. The antiplasmodial activity was determined by comparing the mean parasite clearance in the treated groups, to the negative control group. Also the effect of the extract on the blood packed-cell volume of mice (PCV) was determined.

Results: The LD_{50} of MPR was found to be > 5000 mg/kg. Glycosides, saponins, tannins, flavonoids, triterpenes and alkaloids were the phytochemicals identified in the extract. The extract of MPR produced a significant reduction in the mean parasitemia level compared to the negative control in the curative test: MPR 250 (68.31%, $P < 0.001$), MPR 500 (76.53%, $P < 0.001$), and MPR1000 (83.65%, $P < 0.001$). The extract prolonged the survival of infected mice (18.8 days), compared to the negative control (5.2 days). The extract produced significant chemosuppression compared to the negative control; MPR 250 (73.79%, $P < 0.001$), MPR 500 (81.33%, $P < 0.001$), and MPR 1000 (78.37%, $P < 0.001$). The extract produced significant chemoprophylaxis compared to the negative control; MPR 250 (68.5%, $P < 0.001$), MPR 500 (58.7%, $P < 0.001$), and MPR 1000 (84.77%, $P < 0.001$). The extract was found to have no significant effect on the blood PCV of the treated groups compared to the negative control.

Conclusions: The study showed that the MPR extract has significant antiplasmodial activity in mice at the doses tested, and could justify the traditional use of the plant in the treatment of malaria in Northern Nigeria.

Keywords: In vivo antiplasmodial, Plasmodium berghei, Piliostigma reticulatum, Mice

Background

Malaria is an infectious disease that is caused by *Plasmodium* parasites (*P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale*, and *P. knowlesi*), transmitted by the bites from an infected female anopheles' mosquito (CDC 2020). *P. falciparum* accounts for the highest number of mortality from the disease (CDC 2020). Malaria is a major public health challenge with 229 million cases and 409,000

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deaths reported globally in the year 2019, and Africa has contributed 94% (215,000 million) of the reported cases (WHO 2020). In Africa, Nigeria has the highest burden of the disease, (contributing 25% of the global cases and 27% of global mortality from the disease) in 2019 (WHO 2020).

The advent of malaria parasite resistance to antimalarial drugs is another setback to eradicate the disease. The quinoline-based quinine and later chloroquine proved to be successful treatments for malaria, until quinoline resistance emerged and spread across much of the globe (CDC 2014). Artemisinin proved to be a fast-acting and efficacious remedy against chloroquine-resistant malaria in the face of this circumstance. However, artemisinin resistance in the form of delayed parasite clearance is already on the horizon (White et al. 2014), raising fears that humanity will be left without an effective malaria medication. This necessitates a continuous search for new antimalarial drugs.

Despite pharmaceutical corporations' recent breakthroughs in rational drug design and synthetic chemical techniques, natural products (particularly medicinal plants), have remained a significant source of novel medications (Kaushik et al. 2011). Classes of artemisinins and quinines (which originated from medicinal plants), stand out as the most effective drugs used in modern medicine to treat the malaria menace today. Perhaps the growing concerns of antimalarial drug resistance in certain areas, can be addressed by traditional medicines (Willcox and Bodeker 2004).

Piliostigma reticulatum belongs to *Fabaceae* family and *Caesalpinioideae* subfamily. It is a tree well distributed in the tropics, especially in northern Nigeria, popularly known as "camel's foot" and locally known as "kargo" or "kalgo." The leaves and bark are used in the treatment of malaria, smallpox, neuralgia, dysentery, diarrhea and rheumatism (Zerbo et al. 2010).

Methods

Collection of plant materials

Fresh leaves of *Piliostigma reticulatum* were collected from Ungogo Local Government area of Kano State, Nigeria in January 2018. The botanical identification and authentication were done by a botanist; Baha'uddeen Said Adam at the Herbarium Unit of the Department of Plant Biology, Bayero University Kano. A voucher specimen number; BUKHAN 0072 was deposited at the herbarium for future use.

Extraction of plant materials

Fresh leaves of *Piliostigma reticulatum* were air-dried. The leaves were size reduced into a fine powder using pestle and mortar. One thousand grams (1000 g) of the plant material were macerated with 4 L of 70% methanol for 72 h, with frequent stirring to facilitate the extraction process. After 72 h, the mixture was filtered using Whatman filter paper number 1. The filtrate was concentrated using a water bath at 45 °C and a percentage yield of 10.41% $^{w}/_{w}$ was obtained. The extract was packed in airtight containers, protected from light and stored in a desiccator.

Preliminary phytochemical screening

The phytochemical screening of methanol leaf extract of *Piliostigma reticulatum* was done using standard procedures (Sofowora 1993; Trease and Evans 2002).

Experimental animals

Swiss albino mice of either sex weighing 18–25 g were obtained and maintained in the Animal House, Department of Pharmacology and Therapeutics, Faculty of Pharmaceutical Sciences, Bayero University Kano. The animals were kept based on the guidelines of the National Institutes of Health (NIH 2011).They were fed with standard animal feeds and allowed free access to water ad libitum.

Ethical approval was obtained from the College of Health Sciences, Bayero University Kano, with the Protocol no: BUK/CHS/REC/VII/45.

Rodent Plasmodium parasite

Chloroquine-sensitive *Plasmodium berghei* (*P. berghei*) NK65 strain was used to induce malaria in the experimental mice. The parasite was obtained from the National Institute of Medical Research (NIMR), Yaba, Lagos State, Nigeria. It was maintained via intraperitoneal (*i.p*) sub-passage in mice.

Parasite inoculation

Parasitized blood was collected from donor mouse with rising parasitemia (34%) retro-orbitally. The blood was diluted with 0.9% saline in EDTA containing sample bottle, such that every 0.2 ml of blood contained 1×10^7 infected erythrocytes. Each mouse used in the study was inoculated with 0.2 ml of infected blood intraperitoneally.

Acute toxicity test

The Median lethal dose (LD_{50}) of the extract was determined using the Lorke method (Lorke 1983). The protocol consists of two phases. In the first phase, three groups of three mice were treated orally with graded doses of the extract at 10, 100 and 1000 mg/kg. The mice were observed for signs of toxicity and/or death for the first 4 h and then over 24 h. In the second phase, three mice were divided into three groups of one mouse each, and treated with 1600, 2900 and 5000 mg/kg of the extract, based

on the outcome of the first phase. The animals were also observed for signs of toxicity and/or death over 24 h. The LD_{50} was calculated as the geometric mean of the lowest lethal dose and that of the highest non-lethal dose.

Antiplasmodial screening

Curative test (Rane's Test)

The method of Ryley and Peters (1970) was employed in this study. The inoculum containing 1×10^7 *P. berghei*-infected RBCs (red blood cells) was injected intraperitoneally (*i.p*) into each of the thirty-six (36) mice on the first day (day 0). The mice were maintained for 72 h (day 0–day 3) for the infection to be established. Afterward, the mice were randomly divided into six groups; Group I (negative control) were given 10 ml/kg of distilled water, groups II–IV were treated with the graded doses of the extract (250, 500 and 1000 mg/kg body weight) and groups V and VI (positive controls) were treated with chloroquine 10 mg/kg, and artesunate 5 mg/kg, respectively. The treatment was carried out daily via oral route for 5 days (day 3–day 7). Twenty-four hours after the last treatment, blood was collected from the tail of each mouse and smeared onto a microscope slide to make a thin film. The blood films were fixed with methanol, stained with 10% Giemsa, and examined microscopically to determine the mean parasitemia level (by counting the number of parasitized RBCs in three random microscopic fields).

The Mean Survival Time (MST) for animals in the curative test group, was also determined by calculating the average survival time (in days) of mice after infection with *P. berghei* over a period of 28 days (i.e., from d 0 to d 27), as given by the formula below;

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

Peter's 4-day suppressive test

This test was conducted as described by Peters (1965). Thirty-six (36) mice were infected with *P. berghei* inoculum (1×10^7) *i.p* and distributed into six groups; Group I (negative control) were treated with 10 ml/kg of distilled water (negative control), groups II–IV were treated with graded doses of the extract (250, 500 and 1000 mg/kg), groups V and VI (positive controls) were treated with chloroquine 10 mg/kg and artesunate 5 mg/kg, respectively. Treatment with the extract commenced 4 h after infection (i.e., on Day 0) and continued daily for four days (i.e., from day 0 to day 3). On the 5th day (i.e., day 4), the blood was collected from the tail of each mouse and the parasitemia level was determined microscopically, as done with the curative test.

Prophylactic test (Repository test) This test was done as described by Peters (1965). Thirty mice (30) were randomly distributed into 5 groups. In this test, thirty mice were used as only one positive control was employed (i.e., Pyrimethamine). Group I were treated with 10 ml/kg of distilled water (negative control), groups II-IV were treated with graded doses of the extract (250, 500 and 1000 mg/kg) and group V was treated with Pyrimethamine 1.2 mg/kg (positive control). Treatment continued orally for five days (from day 0 to day 4). On the 6th day (i.e., day 5), the mice were inoculated intraperitoneally with 1×10^7 *P. berghei* infected erythrocytes. After 72 h, blood was collected from the tail of each mouse and the parasitemia level was determined.

Packed cell volume (PCV)

The packed cell volume (PCV) of mice in the curative test group was measured to predict the effectiveness of the extract in preventing hemolysis due to a rise in parasitemia caused by the infection. Blood samples were collected in heparinized capillary tubes by tail bleeding each mouse, and each capillary tube was filled up to ¾ of its length. The end of the capillary tubes was sealed with sealing clay. The tubes were placed in a microhematocrit centrifuge (Sigma-Aldrich, India) with the sealed end of the capillary tubes facing outwards. The blood was centrifuged for 5 min at 11,000 revolutions per minute (rpm). After centrifuging, the capillary tubes were removed from the machine, the PCV was measured using a microhematocrit reader, and the results were recorded. The PCV was calculated using the formula below;

$$\text{PCV} = \frac{\text{Volume of erythrocytes in blood} \times 100}{\text{Total volume of blood}}$$

In this study, the PCV was determined during the curative test on the third day i.e., the day the infection was expected to be established and on the seventh day i.e., after treatment.

Euthanization of mice

The mice used in the study were euthanized as described by Jung et al. 2019. The mice were injected intraperitoneally with Ketamine at 100 mg/kg and Xylazine at 10 mg/kg. Peak serum ketamine level is achieved 10–20 min after the injection (Jung et al. 2019). Therefore, the mice were monitored within that time, until they were unconscious.

Statistical analysis

The statistical analysis was done using SPSS Statistics for Windows, version 16.0 (SPSS Inc., Chicago, Ill., USA). Data were presented as mean ± standard error of mean (mean ± SEM). The difference in mean among the

different groups was analyzed using One-way Analysis of Variance (*ANOVA*) followed by Dunnett's *post hoc* test. Statistical significance was set at $P \leq 0.05$.

Results

Acute toxicity test

The oral median lethal dose (LD_{50}) of methanol leaf extract of *Piliostigma reticulatum* in mice was estimated to be greater than 5000 mg/kg body weight.

Preliminary phytochemical screening

The preliminary phytochemical screening of methanol leaf extract of *Piliostigma reticulatum* revealed the presence of glycosides, saponins, tannins, flavonoids, triterpenes, and alkaloids.

Curative test

The methanol leaf extract of *Piliostigma reticulatum* produced a significant dose-dependent antiplasmodial effect, compared with the distilled water group ($P < 0.001$). At doses of 250 500 and 1000 mg/kg, the extract produced parasite clearance of 68.31%, 76.53% and 83.65%, respectively. The standard drugs chloroquine (10 mg/kg) and artesunate (5 mg/kg) produced parasite clearance of 89.09% and 91.13%, respectively.

The mice in the extract-treated groups survived longer than those in the negative control group; those treated with MPR 1000 mg/kg survived for 19 days, while those in the negative control group survived for 5 days only. The mice in the chloroquine and artesunate-treated groups survived for 26 and 27 days, respectively (Table 1).

Suppressive test

The methanol leaf extract of *Piliostigma reticulatum* demonstrated significant chemo-suppressive activity ($P < 0.001$) as compared with distilled water group. The extract at 250, 500 and 1000 mg/kg had a suppressive effect of 73.79%, 81.33% and 78.37%, respectively. The standard drugs chloroquine 10 mg/kg and artesunate 5 mg/kg, had 86.01% and 87.58% chemo-suppression, respectively (Fig. 1).

Table 1 The curative effect of methanol leaf extract of *Piliostigma reticulatum* against *P. berghei* in mice ($n = 6$)

Treatment (mg/kg)	Mean % parasitemia	Parasite clearance (%)	Mean survival time (Days)
DW 10 ml/kg	82.72 ± 7.30	–	5.2 ± 1.17
MPR (250)	26.21 ± 1.67*	68.31	16.5 ± 1.38*
MPR (500)	19.42 ± 1.04*	76.53	17.3 ± 0.52*
MPR (1000)	13.52 ± 1.70*	83.65	18.8 ± 2.56*
Chloroquine (10)	9.02 ± 1.59*	89.09	26.2 ± 0.75*
Artesunate (5)	7.34 ± 1.69*	91.13	27.3 ± 0.52*

Results are expressed as mean ± SEM. $n = 6$

DW, distilled water; MPR, *Piliostigma reticulatum* methanol extract

* Significantly different from control at $P < 0.001$ using One-way ANOVA and Dunnett's post hoc test

Prophylactic test

The *Piliostigma reticulatum* extract showed significant activity as compared with the distilled water group ($P < 0.001$). At the doses of 250 mg/kg, 500 mg/kg and 1000 mg/kg, the extract produced a chemoprophylactic effect of 68.5%, 58.70% and 84.77%. The standard drug Pyrimethamine at 1.2 mg/kg had a chemoprophylactic effect of 80.7% (Fig. 2).

Packed cell volume (PCV)

The *Piliostigma reticulatum* methanol extract produced no significant effect on the packed cell volume of the extract-treated mice on days 3 and 7 as compared with the distilled water group (i.e., $P > 0.05$). A similar result applies to the chloroquine-treated group. A statistical significance was however observed on day 7 amongst the artesunate-treated group ($P < 0.05$), which had a higher PCV compared with the other groups (Fig. 3).

Discussion

The LD_{50} of *Piliostigma reticulatum* was found to be > 5000 mg/kg, this indicates that the plant is practically non-toxic and therefore safe to the mice (Lorke 1983; Aliyu et al. 2015). This result is similar to the findings of Dosso et al. (2012) and Dosso et al. (2014). Related results were also reported by Ajayi et al. (2019) and Fayanju et al. (2021).

The antiplasmodial activity of the plant could be due to the presence of pharmacologically active principles (glycosides, saponins, tannins, flavonoids, triterpenes, and alkaloids), identified in the plant. Our findings could be supported by earlier studies that reported that alkaloids, flavonoids, triterpenoids, glycosides and tannins possess antiplasmodial activities (Kirby et al. 1989; Phillipson and Wright 1991; Christensen and Kharazmi 2001; Saxena et al. 2003; Willcox and Bodeker 2004; Inbaneson et al. 2012). Further studies supporting our data that the phytochemicals present in *Piliostigma reticulatum* have antiplasmodial activity, includes; Ahmed et al. (2010), Ettebong et al. (2015), Nardos and Makonnen (2017), Onyegbule et al. (2019), Uzor (2020), Abdullahi et al. (2021), Ayisi et al. (2021) and Thakur and Kumari (2021).

The mechanism of antiplasmodial activity of this plant could be explained by the mode of action of the identified secondary metabolites. The plant contains alkaloids, which was reported to act by inhibiting heme transformation into hemozoin in the parasite food vacuole (as

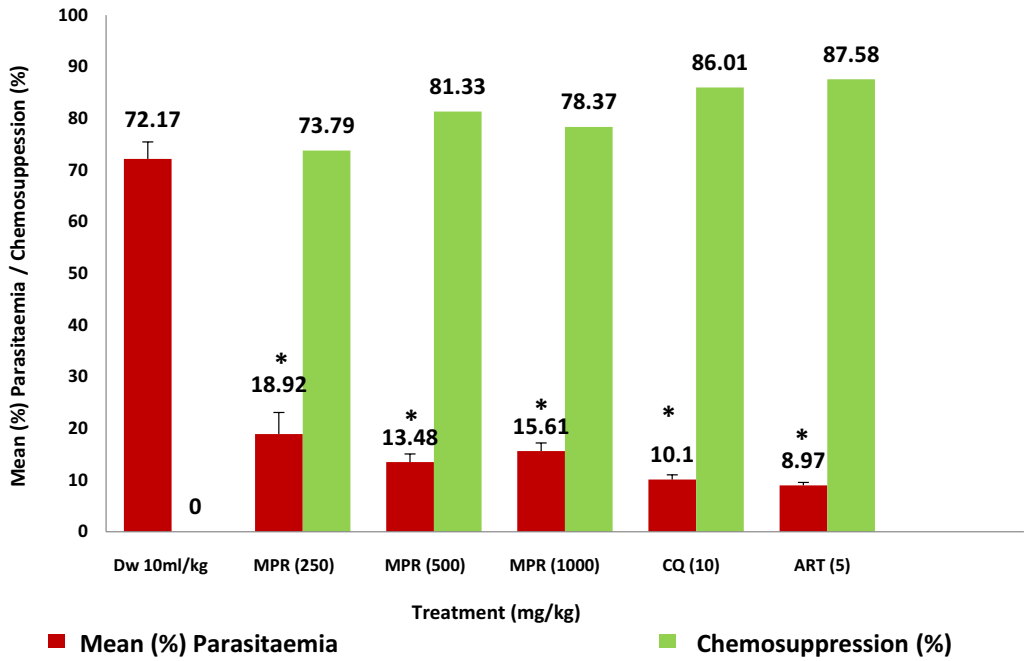

Fig. 1 Suppressive effect of *Piliostigma reticulatum* methanol extract in *P. berghei* infected mice. Results are expressed as mean ± SEM. $n = 6$, *Significantly different from control at $P < 0.001$ using One-way ANOVA and Dunnett's post hoc test. DW, distilled water; MPR, *Piliostigma reticulatum* methanol extract; CQ, chloroquine; ART, artesunate

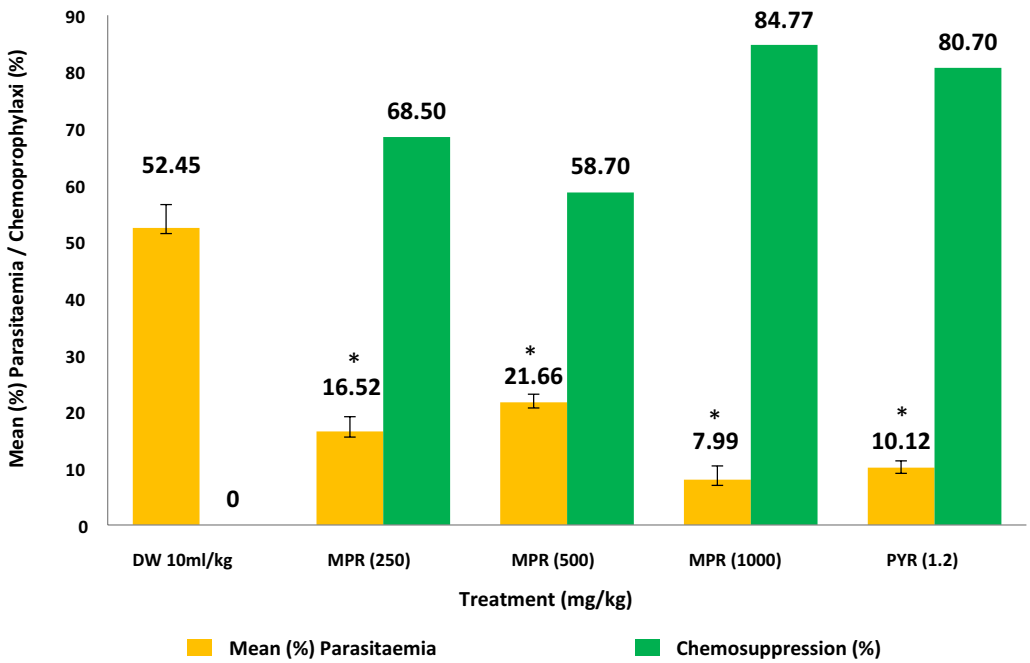

Fig. 2 Prophylactic effect of *Piliostigma reticulatum* methanol leaf extract in *P. berghei* infected mice. Results are expressed as mean ± SEM. $n = 6$, *Significantly different from control at $P < 0.001$ using One-way ANOVA and Dunnett's post hoc test. DW, distilled water; MPR, *Piliostigma reticulatum* methanol extract; PYR, pyrimethamine

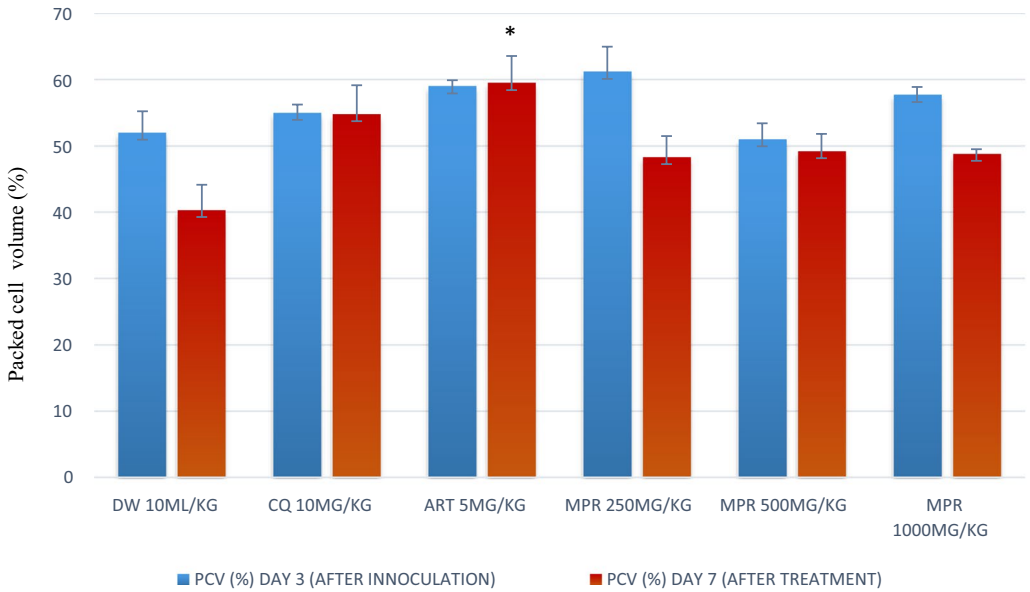

Fig. 3 Effect of *Piliostigma reticulatum* leaf extract on packed cell volume (PCV) of *P. berghei* infected mice in curative test. Results are expressed as mean ± SEM. $n = 6$, *Significantly different from control at $P < 0.05$ using One-way ANOVA and Dunnett's post hoc test. DW, distilled water; MPR, *Piliostigma reticulatum* methanol extract; CQ, chloroquine; ART, artesunate

observed by the quinoline antimalarials) (Inbaneson et al. 2012). Several alkaloids of different classes (Bisindole, indole, terpenoidal, quinolone, etc.) have been identified in medicinal herbs, to possess weak, moderate, and significant antiplasmodial activity (Uzor 2020). Generally, the terpenes (monoterpenes and sesquiterpenes e.g., artemisinins) exert their activity through heme cleavage of the endoperoxide bridge (Okokon et al. 2017). Also, the triterpenes act via inhibition of protein synthesis (Kirby et al. 1989; Inbaneson et al. 2012).

Furthermore, the antimalarial actions of the plant could be due to the flavonoids. This bioactive metabolite interferes with protein biosynthesis and chelates the nucleic acid base pairing of the plasmodium parasite (Okokon et al. 2017; Abdussalam et al. 2018). Flavonoids also inhibit the formation of hemozoin from heme released by digestion of hemoglobin, generating free radicals which elicit the death of the parasites (Marliana et al. 2018). Therefore, the antiplasmodial activity of *Piliostigma reticulatum* extract could be due to the presence of phytochemicals acting singly or in combination to produce a lethal effect on the parasite.

The results of the antiplasmodial screening in this study showed that the methanol extract of *Piliostigma reticulatum* produced significant activity in an established plasmodial infection (curative model), suppressed early form of the plasmodial infection (suppressive model) and protected the mice from the plasmodial infection (prophylactic model).

The mean survival time (MST) is another relevant tool for measuring the activity of antiplasmodial agents. The literature consistently demonstrates that an agent that extends the survival of experimental animals beyond 12 days is considered an effective antiplasmodial agent (Peter and Anatoli 1998; Ural et al. 2014; Widyawaruyanti et al. 2017; Mulisa et al. 2018; Abdullahi et al. 2021). The extract of *Piliostigma reticulatum* achieved a mean survival time of up to 18.8 days, thus, confirming the plants antiplasmodial activity.

The packed cell volume (PCV) was evaluated to measure if the extract can prevent hemolysis in infected animals. Malaria infection is associated with lysis of erythrocytes which could result in anemia, especially in areas of high malaria transmission (Sumbele et al. 2015). Also, some antimalarials may affect the packed cell volume. A drug such as primaquine causes hemolysis (Braga et al. 2015), while therapy with artesunate results in post-artemisinin delayed hemolysis a few days after use (PADH) (Salehi et al. 2018). The case of PADH has been reported with artemether and oral artemisinins (White 2018). In this study, the PCV result showed no significant difference between the extract-treated groups and the control. The phytochemical saponin may be responsible for the reduction in red blood cells caused by the extract (evident from the PCV result). Saponins cause eryptosis by increasing calcium permeability into cells, causing cell shrinkage and cell membrane scrambling, thereby affecting the red blood cells (Bissinger et al. 2014).

Conclusions

The results of our study suggest that the methanol extract of *Piliostigma reticulatum* leaves exhibited antiplasmodial activity against *P. berghei* in mice, at the doses tested, by significantly suppressing parasitemia levels and prolonging the survival of mice. This justifies the ethnomedicinal use of *Piliostigma reticulatum* in the management of malaria in the community.

Abbreviations

ANOVA: Analysis of variance; ART: Artesunate; CDC: Centers for Disease Control and Prevention; CQ: Chloroquine; DW: Distilled water; LD_{50}: Lethal dose 50; MPR: Methanol leaf extract of *Piliostigma reticulatum*; *i.p*: Intraperitoneal; MST: Mean survival time; NIH: National Institutes of Health; NIMR: National Institute of Medical Research; *P. berghei*: *Plasmodium berghei*; *P. falciparum*: *Plasmodium falciparum*; *P. vivax*: *Plasmodium vivax*; *P. malariae*: *Plasmodium malariae*; *P. ovale*: *Plasmodium ovale*; *P. knowlesi*: *Plasmodium knowlesi*; PADH: Post-artemisinin delayed hemolysis; PCV: Packed cell volume; PYR: Pyrimethamine; RBCs: Red blood cells; RPM: Revolutions per minute; SEM: Standard error of mean; WHO: World Health Organization.

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Author contributions

Author SM made the study design. Author SM and SSA collected the plant sample and conducted the laboratory work. Author LAB supervised the laboratory work. Author SM, SSA, USA carried out the literature search. Author SSA, IJA and BA carried out the data analysis. All authors (SM, SSA, IJA, LAB, USA, BA) contributed to writing the manuscript, and each author read and approved the final manuscript.

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Availability of data and materials

All the data generated or analyzed during the study are included in this published article.

Declarations

Ethics approval and consent to participate

The mice used for the study were maintained based on the guidelines of the National Institutes of Health guide for the care and use of laboratory animals (8th edition). The study protocol was approved by the Ethical Committee of the College of Health Sciences, Bayero University Kano with Protocol no: BUK/CHS/REC/VII/45.

Consent for publication

Not applicable.

Competing interests

The authors declare that there are no competing interests regarding this work.

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