

Chemo Suppressive and Curative Potential of *Hypoestes forskalei* Against *Plasmodium berghei*: Evidence for in vivo Antimalarial Activity

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Background: The emergence of drug resistance together with the global burden of malaria triggers the necessity for the searching of new antimalarial agents. This study, therefore, was initiated to investigate the in vivo antimalarial activity of *Hypoestes forskalei* in mice based on the strong supported evidence from the ethnobotanical claims and the in vitro anti-plasmodial activity of the plant.

Methods: The 4-day suppressive (crude extract and fractions) and the Rane's (n-butanol fraction) tests were used to evaluate the antimalarial activity of the plant. A cold maceration technique with 80% methanol was used for the crude extraction of the plant. The crude extract was then fractionated using solvents of different polarity (chloroform, n-butanol, and water).

Results: All the test doses of the crude extract as well as the fractions reduced parasitemia and prolonged mean survival time significantly ($P < 0.001$) as compared to their negative control groups. Maximum parasitemia suppression effect (56%) was observed at the highest dose (600 mg/kg) of the crude extract during the 4-day suppressive test. Likewise, the n-butanol, chloroform, and aqueous fractions showed a percentage suppression of about 50, 38, and 19, respectively, at the dose of 600 mg/kg. Therefore, the n-butanol fraction showed the highest parasitemia suppression followed by the chloroform fraction and then the aqueous fraction. Moreover, the n-butanol fraction showed a significant curative effect ($P < 0.001$) in Rane's test with a percentage suppression of about 49 at a dose of 600 mg/kg.

Conclusion: The study has revealed that the plant has a promising antimalarial activity, the activity being more in the crude extract than the fractions. The highest antimalarial activity of the n-butanol fraction suggests that non-polar and medium polar principles could be responsible for the observed activity.

Keywords: antimalarial activity, *Plasmodium berghei*, *Hypoestes forskalei*, parasitemia

Background

Malaria, the most common and prevalent mosquito-borne disease, is caused by five *Plasmodium* species. Indeed, most malaria infections worldwide are caused by the two species, namely: *Plasmodium falciparum* (*P. falciparum*) and *Plasmodium vivax* (*P. vivax*).¹ Malaria remains one of the top challenging health problems globally. As evidence, an estimated 228 million cases (213 million in Africa) and 405 thousand deaths (380 thousand in Africa) of malaria occurred worldwide in 2018 only. Despite much progress, malaria remains one of the top challenging health problems in Africa, since more than 90% of worldwide deaths

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occur in this region. Still, the majority of the deaths and associated burdens of malaria in sub-Saharan Africa are due to *P. falciparum*.² In addition, malaria-associated maternal illness and anemia, preterm birth, and low birth-weight newborns occur predominantly in Africa. Children under 5 years, most of which were in sub-Saharan Africa, account for about 70% of all malaria deaths globally.^{2,3}

Over the past half century, malaria parasites, primarily *P. falciparum* and *P. vivax*, have become progressively resistant to antimalarial drugs and insecticides, the key challenge and threat against malaria control and elimination effort.⁴⁻⁶ Therefore, the emergence of drug resistance together with the global burden of malaria triggers the necessity for the searching of new antimalarial agents. As a matter of fact, plants have been used as a source of many drugs including the currently available antimalarial drugs and therefore could be considered as a potential source of new antimalarial drugs.⁷

The study plant, *Hypoestes forskalei* (*H. forskalei*) (Vahl) R, belongs to the genus *Hypoestes* (family *Acanthaceae*) and includes over 150 species. The ethnopharmacological studies of the plant indicate that it possesses various bioactivities such as cytotoxic,^{8,9} antimicrobial,⁹⁻¹¹ larvaicidal,¹² antioxidant,^{9,13} antipyretic,^{9,13} antileishmanial,¹⁴ and antitrypanosomal.¹⁴ Furthermore, it has been used traditionally for different kinds of ailments including malaria.¹⁵

The antimalarial activity of the plant has been supported by various ethnobotanical studies.^{16,17} Furthermore, different in vitro studies also reported that the methanolic extracts of *H. forskalei* possessed antiplasmodial activity against *P. falciparum* with an IC₅₀ of 5.13–8.8 µg/mL.^{14,18,19} Besides, other species of the genus *Hypoestes* were also reported to have promising antimalarial activity in different in vitro studies.^{16,20} This study, therefore, was initiated to investigate the antimalarial activity of the plant in a rodent model of malaria based on the strong supporting evidence from the in vitro antiplasmodial activity together with ethnobotanical claims of *H. forskalei*.

Methods

Plant Material

The leaves of *H. forskalei* were collected from Tigray region, Ethiopia, in October 2019. Identification and authentication of the plant specimen was done by a taxonomist, and a voucher specimen (DM 009/2019) was deposited for future reference at the National

Herbarium, College of Natural and Computational Sciences, Addis Ababa University, for future reference.

Experimental Animals and Parasite

Healthy Swiss albino mice obtained from Wollo University Department of Pharmacy, weighing 20–30 g and aged 6–8 weeks, were used for the experiment. All procedures and techniques used in this study were in accordance with the organization for economic co-operation and development (OECD) guideline 425 and the national research council of the national academies (Institute for Laboratory Animal Research).^{21,22} Besides, the protocol was approved by the Research and Ethics Committee of the department of Pharmacy of Wollo University. The mice were acclimatized to laboratory conditions for 1 week prior to the experiment. The chloroquine sensitive strain of *plasmodium berghei* (*p. berghei*) was used for the antimalarial tests.

Extraction and Solvent Fractionation

After collection, the leaves of *H. forskalei* were dried at room temperature under shade and then crushed into a coarse powder using a mortar and pestle so as to ease the extraction process. About 600 g of the coarse plant material was then extracted by cold maceration technique (100 g of dried leaves in 600 mL of 80% methanol) for 3 days. The extract was first filtered using gauze and then with Whatman filters paper No. 1 (Whatman®, England). The remaining residue was re-macerated for another 72 hours twice and filtered in order to exhaustively extract the possible available bioactive components. Afterwards, the filtrates were concentrated in a rotary evaporator (Buchi type TRE121, Switzerland) and the concentrated filtrate was then frozen in a freezer and dried in a lyophilizer (Wagtech Jouan Nordic DK-3450 Allerod, Denmark).

About 2/3 of the 80% methanol extract (80ME) was fractionated using solvents of different polarity (chloroform, n-butanol, and water) in order to identify in which fraction the active compounds are concentrated. After the crude extract was mixed with water, chloroform was added (three times) to the formed suspension and then shaken in a separatory funnel in order to isolate the chloroform fraction. By the same manner, n-butanol was added to the remaining aqueous residue to separate the n-butanol fraction. Thereafter, further concentration of the n-butanol and chloroform fractions were done in a rotary evaporator. Besides, lyophilization was done for the last aqueous residue to obtain the aqueous fraction. Finally, the crude

extract and fractions were stored in a freezer (-20°C) until used for the experiment.

Acute Toxicity Test

An acute oral toxicity test for 80% methanol extract (80ME) and solvent fractions of the leaves of *H. forskalei* was performed according to the standards.²³ For a sighting study, a dose of 2000 mg/kg was given for a single mouse by oral gavage for each of the 80ME and fractions. Since no death was observed within 24 hours, an additional four mice were used, and administered the same dose of 80ME and fractions. Then, the mice were followed continuously every 30 minutes for 4 hours and then daily for the next 14 days for the general signs and symptoms of toxicity.

Animal Grouping and Dosing

In both models (4-day suppressive and curative test), animals were randomly divided into five groups (negative control, positive control, and three test groups) comprising of six animals per group. The test groups (groups 1, 2, and 3) received different doses (200, 400, and 600 mg/kg, respectively) of 80ME and fractions orally, which were determined based on the acute oral toxicity test and pilot study. The remaining two groups served as negative and positive controls and were administered distilled water (2 mL/100 g) and chloroquine 25 mg/kg, respectively, in both models.²⁴

Inoculation of Mice

Albino mice previously infected with *p. berghei* and having a parasitemia level of 20–30% were used as donors. The donor mice were sacrificed by cervical dislocation, and blood was collected into heparinized capillary tubes containing 0.5% trisodium citrate. The blood was then diluted with normal saline (0.9%) based on parasitemia of the donor mice and the red blood cells (RBC) count of normal mice in such a way that 1 mL blood contained 5×10^7 infected erythrocytes. Thereafter, 0.2 mL blood, containing 1×10^7 *p. berghei* infected erythrocytes, was injected through intraperitoneal (ip) route for each mouse.

Determination of Antimalarial Activity

Four-Day Suppressive Test

The chemo-suppressive activity of the plant was evaluated by the Peter's 4-day suppressive test against mice infected with chloroquine sensitive *p. berghei*.²⁵ Thirty mice were infected on the first day (day 0) for each of 80ME and

solvent fractions. Two-hour post-infection, the mice were randomly distributed into the five groups and treated as described in the Animal Grouping and Dosing section. Afterwards, treatment was continued for an additional 3 consecutive days (until day 3). Determination of parasitemia was conducted on day 4 of the experiment (at 96 hours post-infection). In addition, other parameters like body weight, temperature, and packed cell volume (PCV) were measured just before infection and at the end of the experiment.

Curative Test

The curative potential of the n-butanol fraction was evaluated using Rane's test.²⁶ After 72 hours (day 3) of infection with the parasite, five groups were assigned randomly and treated with their respective doses as described in the Animal Grouping and Dosing section. Treatment was continued for a further 3 days and the parasitemia level was recorded daily until day 7. Likewise, body weight, temperature, and PCV were also measured just before infection and at the end of the experiment.

Determination of Parasitemia and Survival Time

Parasitemia determination was done by counting the number of infected RBCs (a minimum of three fields per slide) using a light microscope (MB23 0 T, China) with an objective lens magnification power of 100x. Percent parasitemia and percent inhibition were calculated using the modified Peters and Robinson formula.²⁷

$$\% \text{ Parasitemia} = \frac{\text{Number of parasitized RBC}}{\text{Total number of RBC count}} \times 100$$

$$\% \text{ Suppression} = \frac{\% \text{ Parasitemia in negative control} - \% \text{ Parasitemia in study group}}{\% \text{ Parasitemia in negative control}} \times 100$$

Finally, the animals were followed and their mean survival time (MST) was determined using the following formula.²⁸

$$\text{MST} = \frac{\text{Total number of days mice survived}}{\text{Total number of mice}}$$

Determination of Packed Cell Volume, Rectal Temperature, and Body Weight

A microhematocrit centrifuge (Hettichhaematokrit, Germany), centrifugation at 12,000 rpm for 5 minutes, was

used for the determination of PCV after blood was collected from the tail of each mouse using heparinized capillary tubes. In addition, body weight and rectal temperature of each mouse were determined using sensitive digital weighing balance and rectal temperature, respectively.

PCV was determined using the following calculation.²⁹

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

Preliminary Phytochemical Screening

The qualitative phytochemical investigations of 80ME and fractions (chloroform, n-butanol, and aqueous) were carried out using standard tests.^{30,31}

Data Analysis

Data was analyzed using windows SPSS version 23.0. Results for the study parameters are expressed as mean \pm standard error of the mean (SEM). One-way ANOVA followed by Tukey's post-hoc test for multiple comparisons was used to compare results among groups. Furthermore, the gradual development of parasitemia across days of treatment in Rane's test was analyzed by two-way repeated measures ANOVA. Finally, the results were deemed significant at 95% confidence level when the *P*-value was <0.05 .

Results

Acute Oral Toxicity Test

The acute oral toxicity test of 80ME and solvent fractions of leaves of *H. forskalei* indicated that neither the 80ME nor the solvent fractions caused gross behavioral changes and mortality within 24 hours as well as in the following days, indicating that the LD50 values of the extract and fractions is greater than 2000 mg/kg in mice.

Table 1 Parasitemia and Survival Time of Infected Mice Treated with 80% Methanol Extract of the Leaves of *Hypoestes forskalei* in the 4-Day Suppressive Test

Groups	% Parasitemia	% Suppression	Survival Time (Days)
2% TW80	37.85 \pm 0.52	–	7.22 \pm 0.25
CQ 25 mg/kg	0.00 \pm 0.00	100.00 ^{a3}	28.00 \pm 0.00 ^{a3}
200 mg/kg	20.22 \pm 0.24	46.58 ^{a3b3d3e3}	11.50 \pm 0.20 ^{a3b3d2e3}
400 mg/kg	18.44 \pm 0.10	51.28 ^{a3b3e3}	12.90 \pm 0.32 ^{a3b3e3}
600 mg/kg	16.62 \pm 0.11	56.00 ^{a3b3}	15.05 \pm 0.25 ^{a3b3}

Notes: Data are expressed as mean \pm SEM; n=6; a, compared to negative control; b, to CQ25 mg/kg; d, to 400 mg/kg; e, to 600 mg/kg; 2, *P*<0.01; 3, *P*<0.001; 2% TW80, 2% Tween80; CQ, chloroquine.

Effect of 80% Methanol Extract in the 4-Day Suppressive Test

The three test doses of the 80ME reduced parasitemia as well as prolonged MST significantly (*P*<0.001) as compared to the negative control group in a dose dependent manner. Similarly, MST was significantly prolonged (*P*<0.001) by all doses of the extract. However, both parasitemia suppression and MST effects were significantly (*P*<0.001) lower than the chloroquine treated groups (Table 1).

The results of PCV and rectal temperature displayed that the crude extract had a substantial dose dependent protective effect (*P*<0.001) as compared to the negative control groups (Table 2). Moreover, the highest dose (600 mg/kg) of the extract showed a significant (*P*<0.05) protective effect in comparison with the lower dose (200 mg/kg) with regard to PCV and rectal temperature. In addition, the middle and higher doses of the extract showed a significant effect (*P*<0.01) in the prevention of body weight reduction as compared to the negative control groups. Nonetheless, a statistically significant effect was not observed among the test doses of the crude extract with regard to the amelioration of body weight (Table 2).

Table 2 Packed Cell Volume, Rectal Temperature, and Body Weight of Infected Mice Treated with 80% Methanol Extract of the Leaves of *Hypoestes forskalei* in the 4-Day Suppressive Test

Groups	Packed Cell Volume			Rectal Temperature			Body Weight		
	D ₀	D ₄	% Change	D ₀	D ₄	% Change	D ₀	D ₄	% Change
2% TW80	60.80 \pm 1.18	56.11 \pm 1.02	–7.71	36.66 \pm 0.16	34.82 \pm 0.14	–5.01	28.20 \pm 0.21	26.85 \pm 0.22	–4.79
CQ25 mg/kg	60.62 \pm 1.23	60.20 \pm 1.15	–0.69 ^{a3}	36.40 \pm 0.23	36.11 \pm 0.31	–0.80 ^{a3}	28.83 \pm 0.32	28.26 \pm 0.29	–1.98 ^{a3}
200 mg/kg	60.58 \pm 0.58	57.60 \pm 0.47	–4.92 ^{a3b3e1}	36.32 \pm 0.11	35.32 \pm 0.15	–2.75 ^{a3b3e1}	28.45 \pm 0.32	27.70 \pm 0.34	–3.65 ^{b3}
400 mg/kg	60.88 \pm 0.70	58.85 \pm 0.53	–3.33 ^{a3b3}	36.40 \pm 0.20	35.66 \pm 0.17	–2.03 ^{a3b3}	28.86 \pm 0.44	27.95 \pm 0.35	–3.15 ^{a2b3}
600 mg/kg	60.90 \pm 0.45	59.50 \pm 0.57	–2.29 ^{a3b3}	36.20 \pm 0.17	35.50 \pm 0.19	–1.93 ^{a3b3}	28.42 \pm 0.54	27.66 \pm 0.66	–2.67 ^{a2b3}

Notes: Data are expressed as mean \pm SEM; n=6; a, compared to negative control; b, to CQ25 mg/kg; e, to 600 mg/kg; 1, *P*<0.05; 2, *P*<0.01; 3, *P*<0.001; 2% TW80, 2% Tween80; CQ, chloroquine; D₀, pre-treatment value on day 0; D₄, post-treatment value on day 4.

Effect of Solvent Fractions on the 4-Day Suppressive Test

Although lower than the standard drug ($P<0.001$), all the test doses of the three fractions reduce parasitemia significantly ($P<0.001$) in comparison with their respective negative control groups. Moreover, comparison among the three fractions showed that the n-butanol fraction showed the highest parasitemia suppression followed by chloroform fraction and then the aqueous fraction (Table 3). Besides, a dose dependent parasitemia suppression effect was also observed among the test doses, ie, the highest dose of all the three fractions had a significant effect with their lowest test doses. Additionally, the dose dependent effect was more pronounced in n-butanol and chloroform fractions, ie, the middle and highest doses of these fractions also showed a statistically significant ($P<0.01$) effect as compared with the lowest test doses (Table 3).

Likewise, all the three fractions prolonged the survival time of parasite infected mice significantly ($P<0.001$) in comparison with the negative control groups, despite the effect still not being lower than the chloroquine treated groups. Besides, a dose-dependent effect ($P<0.001$) was also observed between the highest doses and the lowest doses of the three fractions (Table 3). However, only the middle dose of the n-butanol fraction showed a significant

Table 3 Parasitemia and Survival Time of Infected Mice Treated with Solvent Fractions of the Leaves of *H. forskalei* in the 4-Day Suppressive Test

Groups	% Parasitemia	% Suppression	Survival Time (Days)
2% TW80	37.68±0.66	–	7.45±0.22
CQ25 mg/kg	0.00±0.00	100.00 ^{a3}	28.00±0.00 ^{a3}
200 mg/kg CF	26.32±0.24	30.14 ^{a3b3d2e3}	10.12±0.25 ^{a3b3e1}
400 mg/kg CF	24.36±0.33	35.35 ^{a3b3}	10.74±0.39 ^{a3b3}
600 mg/kg CF	23.48±0.22	37.68 ^{a3b3}	11.11±0.27 ^{a3b3}
2% TW80	37.92±0.61	–	7.18±0.17
CQ25 mg/kg	0.00±0.00	100.00 ^{a3}	28.00±0.00 ^{a3}
200 mg/kg BF	22.35±0.28	41.06 ^{a3b3d2e3}	11.15±0.39 ^{a3b3d2e1}
400 mg/kg BF	20.66±0.35	45.52 ^{a3b3}	12.58±0.23 ^{a3b3e1}
600 mg/kg BF	18.85±0.26	50.29 ^{a3b3}	13.78±0.26 ^{a3b3}
2% TW80	37.88±0.42	–	7.17±0.23
CQ25 mg/kg	0.00±0.00	100.00 ^{a3}	28.00±0.00 ^{a3}
200 mg/kg AF	31.45±0.38	16.97 ^{a3b3e1}	8.87±0.24 ^{a3b3e1}
400 mg/kg AF	30.77±0.25	18.77 ^{a3b3}	9.54±0.26 ^{a3b3}
600 mg/kg AF	30.67±0.26	19.03 ^{a3b3}	9.75±0.22 ^{a3b3}

Notes: Data are expressed as mean±SEM; n=6; CF, chloroform fraction; BF, butanol fraction; AF, aqueous fraction; a, compared to negative control; b, to CQ25 mg/kg; d, to 400 mg/kg; e, to 600 mg/kg; 1, $P<0.05$; 2, $P<0.01$; 3, $P<0.001$; 2% TW80, 2% Tween80; CQ, chloroquine.

effect ($P<0.05$) with its lowest dose treated group (Table 3).

The results of the fractions on three parameters (PCV, rectal temperature, and body weight) during the 4-day suppressive test suggested that all the doses of the fractions had a significant ($P<0.001$) protective effect in comparison with the negative control groups, still the standard drug was more effective than all the fractions ($P<0.001$). In addition, the n-butanol fraction had a more protective effect than the chloroform and aqueous fractions. Besides, a significant dose dependent effect was also observed among the test doses of the n-butanol fraction in prevention of PCV reduction, while the aqueous and chloroform fractions failed to do so (Table 4). Similarly, only the highest dose of n-butanol fraction had a significant dose-dependent effect ($P<0.05$) to its lower dose in prevention of rectal temperature decline (Table 4). In contrast, a dose dependent effect was not appreciated among the test doses of all the fractions in the prevention of body weight reduction, as depicted in Table 4.

Effect of n-Butanol Fraction in the Rane's Test

The n-butanol fraction showed the highest parasitemia suppression during the 4-day suppressive test among the fractions and, hence, its curative potential was evaluated using the Rane's test. Accordingly, all the test doses of the n-butanol fraction showed a significant effect ($P<0.001$) with regard to the curative effect and prolongation of MST as compared to the negative control group, even though the effect was still lower ($P<0.001$) than the standard drug. Definitely, a substantially significant ($P<0.001$) parasitemia development difference was also detected via the two-way repeated measures ANOVA analysis across the course of treatment. In addition, the ongoing increment of parasitemia in the test groups was lower than the negative control group during the course of treatment (Figure 1). Furthermore, a dose dependent effect ($P<0.001$) was also noticed among all the three test doses (Table 5).

Once more, a significant effect ($P<0.001$) was also noticed by all the test doses of the n-butanol fraction in the attenuation of PCV reduction and rectal temperature in a dose-dependent manner as compared to the negative control group (Table 6). However, substantial ($P<0.01$) prevention of body weight reduction was achieved only by the middle and higher doses of the fraction. Besides, a dose-dependent effect was not observed among the three test

Table 4 Packed Cell Volume, Rectal Temperature, and Body Weight of Infected Mice Treated with Solvent Fractions of the Leaves *Hypoestes forskalei* in the 4-Day Suppressive Test

Groups	Packed Cell Volume			Rectal Temperature			Body Weight		
	D ₀	D ₄	% Change	D ₀	D ₄	% Change	D ₀	D ₄	% Change
2% TW80	59.34±0.14	54.07±0.44	-8.88	36.52±0.14	34.64±0.35	-5.14	28.382±0.21	27.072±0.19	-4.61
CQ25 mg/kg	60.15±0.31	59.86±0.23	-0.48 ^{a3}	35.48±0.04	35.49±0.15	0.03 ^{a3}	28.082±0.44	28.422±0.22	1.21 ^{a3}
200 mg/kg CF	59.53±0.13	55.94±0.53	-6.03 ^{a3b3}	36.31±0.11	34.99±0.22	-3.64 ^{a3b3}	28.142±0.09	26.962±0.33	-4.19 ^{a1b3}
400 mg/kg CF	60.01±0.33	56.82±0.44	-5.31 ^{a3b3}	36.20±0.08	35.34±0.65	-2.37 ^{a2b3}	27.972±0.55	26.982±0.45	-3.54 ^{a1b3}
600 mg/kg CF	60.71±0.23	57.82±0.37	-4.76 ^{a3b3}	35.73±0.34	35.00±0.78	-2.04 ^{a2b3}	28.162±0.33	27.212±0.39	-3.37 ^{a2b3}
2% TW80	59.34±0.11	54.04±0.31	-8.93	36.52±0.14	34.59±0.71	-5.28	28.402±0.19	27.072±0.22	-4.68
CQ25 mg/kg	60.15±0.09	59.86±0.18	-0.48 ^{a3}	35.49±0.43	35.49±0.65	0.00 ^{a3}	28.102±0.36	28.422±0.21	1.14 ^{a3}
200 mg/kg BF	59.46±0.23	56.3±0.22	-5.31 ^{a3b3de2}	36.36±0.35	35.06±0.09	-3.57 ^{a1b3e2}	28.012±0.39	26.852±0.19	-4.14 ^{a1b3}
400 mg/kg BF	60.06±0.53	57.56±0.51	-4.16 ^{a3b3}	36.25±0.22	35.43±0.55	-2.26 ^{a3b3}	27.972±0.32	27.022±0.18	-3.40 ^{a1b3}
600 mg/kg BF	60.76±0.33	58.52±0.27	-3.69 ^{a3b3}	35.78±0.33	35.11±0.39	-1.87 ^{a3b3}	27.662±0.52	26.802±0.23	-3.11 ^{a2b3}
2% TW80	59.27±0.43	54.05±0.63	-8.70	36.5±0.24	34.59±0.44	-5.23	28.402±0.29	27.072±0.33	-4.68
CQ25 mg/kg	60.13±0.36	59.86±0.23	-0.45 ^{a3}	35.49±0.55	35.48±0.67	-0.03 ^{a3}	28.102±0.18	28.422±0.22	1.14 ^{a3}
200 mg/kg AF	59.58±0.55	55.01±0.17	-7.67 ^{a3b3}	36.38±0.08	34.95±0.77	-3.93 ^{a1b3}	28.172±0.35	26.972±0.29	-4.26 ^{b3}
400 mg/kg AF	60.05±0.73	55.7±0.39	-7.24 ^{a3b3}	36.24±0.55	35.04±0.23	-3.31 ^{a2b3}	28.012±0.36	26.822±0.24	-4.25 ^{b3}
600 mg/kg AF	60.69±0.43	56.52±0.53	-6.87 ^{a3b3}	35.83±0.78	34.91±0.19	-2.57 ^{a2b3}	28.232±0.56	27.122±0.22	-3.93 ^{b3}

Notes: Data are expressed as mean±SEM; n=6; CF, chloroform fraction; BF, butanol fraction; AF, aqueous fraction; a, compared to negative control; b, to CQ25 mg/kg; e, to 600 mg/kg; 1, P<0.05; 2, P<0.01; 3, P<0.001; 2% TW80, 2% Tween80; CQ, chloroquine; D₀, pre -treatment value on day 0; D₄, post-treatment value on day 4.

doses with regard to body weight reduction and still the standard drug was superior ($P<0.001$) than the test doses.

Preliminary Phytochemical Screening

The preliminary phytochemical screening of 80ME revealed the presence of all tested constituents except tannins and saponins. Polyphenols, terpenoids, alkaloids, steroids, and flavonoids were detected in both chloroform and n-butanol fractions, whereas polyphenols, terpenoids, and alkaloids were detected in aqueous fraction (Table 7).

Discussion

Due to the possible prodrug effect and involvement of the immune system in eradication of infection, an in vivo model was adopted in this study.³² The acute oral toxicity result of this study inferred that the oral medial lethal dose (LD50) of the plant might be greater than 2000 mg/kg, justifying its safety for antimalarial use, as per OECD guideline No 425.²³

The potential antimalarial activity of the crude extract as well as the fractions were evaluated by the 4-day suppressive test, a standard model for the antimalarial screening.³³ Accordingly, all the test doses of the crude extract as well as the fractions inhibited the level of parasitemia and improved the survival time of infected mice in a dose dependent manner. Therefore, the plant could have

the potential of schizontocidal activity in early infection at which the primary attack due to malaria can be controlled.²⁵

Concerning the fractions, maximum parasitemia suppression and prolongation of MST was attained by the n-butanol fraction followed by the chloroform fraction, implying that the most active principles are more concentrated in these fractions (Figure 1).³⁴ Besides, these findings, i.e, the highest and least chemo-suppressive effects of n-butanol and aqueous fractions, respectively, are concordant with some findings.^{29,35} These observations further reinforce the notion that compounds responsible for anti-malarial activity of the plant might be concentrated in non-polar and semi-polar fractions.³⁶ According to the 4-day chemo-suppressive test, the fractions exhibited lower parasitemia suppression and MST than that of the crude extract. Accordingly, the higher antimalarial activity of the crude extract than the fractions could be due to less concentration and loss of synergistic activity of active ingredients upon fractionation,²⁹ as supported by different similar studies.^{7,29,37}

As described above, the n-butanol fraction was the one with the highest parasitemia suppression during the 4-day suppressive test and therefore its curative potential was further evaluated through Rane's test. Although the percentage suppression was relatively lower than its effect in the 4-day suppressive test, the n-butanol fraction still

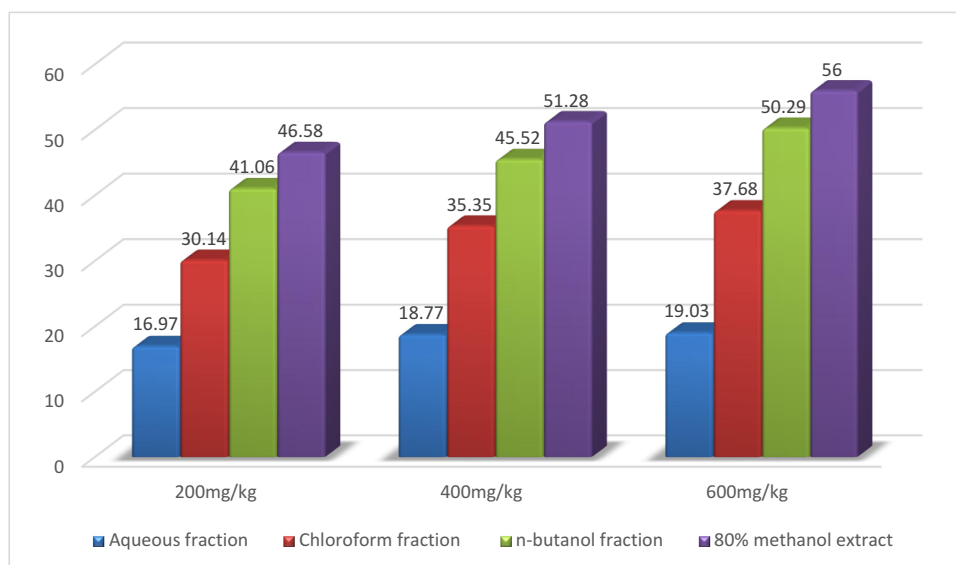


Figure 1 Comparison of percentage suppression of the crude and solvent fractions of the leaves of *Hypoestes forskalei* in 4-day suppressive test.

displayed a promising curative potential in established infection. Justifying the probable rapid action of the extract during the Rane's test,³⁸ its parasitemia suppression effect started after the first dose as compared to the chloroquine treated group (Figure 2). The overall higher suppressive effect than curative effect of the n-butanol fraction is in agreement with other studies where bioactive compounds had a more profound effect on early infection than established infection.^{39,40} Taken together, the results of the Rane's test imply that the n-butanol fraction has therapeutic potential against established malaria infection since it is desirable to have both suppressive and curative activities in a phytodrug.⁴¹

In both models (4-day suppressive test and Rane's test), percentage parasitemia suppression and survival time prolongation are the most reliable parameters³³ and

Table 5 Parasitemia and Survival Time of Infected Mice Treated with n-Butanol Fraction of the Leaves of *Hypoestes forskalei* in Rane's Test

Groups	% Parasitemia	% Suppression	Survival Time (Days)
2% TW80	71.65±0.43	–	7.58±0.22
CQ25 mg/kg	0.00±0.00	100.00 ^{a3}	28.00±0.00 ^{a3}
200 mg/kg	46.55±0.71	35.03 ^{a3b3d3e3}	11.16±0.30 ^{a3b3d1e3}
400 mg/kg	42.24±0.64	41.05 ^{a3b3e3}	12.50±0.34 ^{a3b3e2}
600 mg/kg	36.65±0.35	48.85 ^{a3b3}	13.17±0.31 ^{a3b3}

Notes: Data are expressed as mean±SEM; n=6; a, compared to negative control; b, to CQ25 mg/kg; d, to 400 mg/kg; e, to 600 mg/kg; 1, P<0.05; 2, P<0.01; 3, P<0.001; 2% TW80, 2% Tween80; CQ, chloroquine.

compounds are considered active when reduction in parasitemia is $\geq 30\%$.⁴² Consequently, the crude extract as well as both the chloroform and n-butanol fractions were presumed to be active. Supporting evidence for this assertion come from the ethnobotanical claims as well as the in vitro antimalarial studies of *H. forskalei*.^{14,18,19}

According to the phytochemical screening analysis, the crude extract and the fractions of the leaf of *H. forskalei* are rich in many secondary metabolites including polyphenols, flavonoids, terpenoids, steroids, alkaloids, and glycosides, and it is in agreement with previous studies done on *H. forskalei*.^{12,19,20,43} Although a bio-guided fractionation of the active compound is not identified in this study, the anti-malarial activity of *H. forskalei* could be accredited to a single or a combination of its bioactive compounds.^{44,45} Interestingly, a recent in vitro study revealed the isolation of a promising antimalarial compound, 15b-hydroxycryptopleurine-N-oxide, from *H. forskaolii* using bio-guided fractionation procedures.⁴³ Similarly, moderately active in vitro antimalarial fusicoccane diterpenes were previously isolated from the methanolic extract of the aerial parts of *H. forskalei*.¹⁹ Therefore, based on the strong supporting evidences from the previous in vitro and ethnobotanical studies of this plant as well as from this study, the observed antimalarial activity of *H. forskalei* could be ascribed to the presence of secondary metabolites like alkaloids, terpenoids, flavonoids, phenols, and other compounds.^{46,47}

Bioactive compounds found in the crude extract and solvent fractions of the plant could produce their antimalarial effect via different mechanisms. Thus, flavonoids

Table 6 Packed Cell Volume, Rectal Temperature, and Body Weight of Infected Mice Treated with n-Butanol Fraction of the Leaves of *Hypoestes forskalei* in Rane's Test

Groups	Packed Cell volume			Rectal Temperature			Body Weight		
	D ₃	D ₇	% Change	D ₃	D ₇	% Change	D ₃	D ₇	% Change
2% TW80	51.66±0.36	43.00±0.33	-16.76	35.23±0.24	33.38±0.25	-5.25	28.29±0.45	25.56±0.52	-9.65
CQ25 mg/kg	50.11±0.32	51.64±0.25	3.05 ^{a3}	35.05±0.21	35.34±0.20	0.83 ^{a3}	28.49±0.52	28.93±0.43	1.54 ^{a3}
200 mg/kg	51.73±0.22	46.42±0.18	-10.26 ^{a3b3d2e3}	35.18±0.15	33.70±0.18	-4.20 ^{a3b3d1e3}	26.86±0.33	24.57±0.25	-8.52 ^{b3}
400 mg/kg	51.64±0.25	47.66±0.22	-7.70 ^{a3b3}	35.23±0.35	33.92±0.31	-3.72 ^{a3b3}	28.08±0.43	25.9±0.42	-7.76 ^{a2b3}
600 mg/kg	51.58±0.06	48.06±0.17	-6.82 ^{a3b3}	35.23±0.19	34.1±0.21	-3.20 ^{a3b3}	27.49±0.53	25.45±0.32	-7.42 ^{a2b3}

Notes: Data are expressed as mean±SEM; n=6; a, compared to negative control; b, to CQ25 mg/kg; d, to 400 mg/kg; e, to 600 mg/kg; 1, P<0.05; 2, P<0.01; 3, P<0.001; 2% TW80, 2% Tween80; CQ, chloroquine; D₃, pre-treatment value on day 3; D₇, post-treatment value on day 7.

Table 7 Preliminary Phytochemical Screening of 80% Methanol Extract and Solvent Fractions of the Leaves of *Hypoestes forskalei*

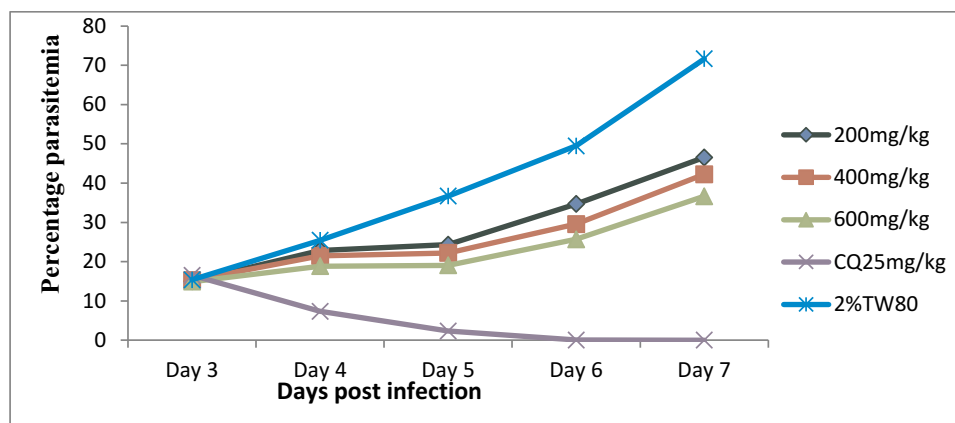
Metabolites	80% Methanol Extract	Solvent Fractions		
		Chloroform Fraction	N-butanol Fraction	Aqueous Fraction
Polyphenols	+	+	+	+
Terpenoids	+	+	+	+
Flavonoids	+	+	+	-
Steroids	+	+	+	-
Alkaloids	+	+	+	+
Glycosides	+	-	+	-
Tannins	-	-	-	-
Saponins	-	-	-	-

Notes: + = presence, - = absence.

and steroidal compounds have been found to inhibit the growth and multiplication of the parasite by preventing the influx of essential nutrients, through different molecular mechanisms, that are essential for the parasite.^{48,49} Likewise, phytochemicals like sesquiterpenes, alkaloids, and polyphenols could have exerted their action by cytotoxic effect on the parasites.^{46,47,49,50} Bioactive compounds may also prevent parasitic invasion through

modulating the membrane properties of the erythrocytes.⁵¹ Besides, phytochemicals like steroids, flavonoids, glycosides, and others may also exert their anti-malarial effects indirectly by stimulating the immune system of the host.^{52,53}

PCV and temperature reduction as well as body weight loss are the cardinal signs of malaria-infected mice.⁵⁴ Therefore, a good antimalarial agent is expected to mitigate

**Figure 2** Parasitemia development over the course of treatment with n-butanol fraction of the leaves of *Hypoestes forskalei* in Rane's model.

a drop in these parameters due to the rise in parasitemia.⁵⁵ Indeed, the crude extract and the three fractions (in the 4-day suppressive test) as well as the n-butanol fraction (in the curative test) avert the reduction in PCV of parasite infected mice. This implies the effectiveness of the crude extract and solvent fractions of the leaf of *H. forskalei* in reducing malaria-induced hemolysis together with its parasitemia suppression activity.⁵⁵ Furthermore, the antioxidant effect of *H. forskalei* could have an additional protective mechanism for RBCs against oxidative stress that arise during infection and, hence, might increase their survival.^{9,13}

Malaria infection in mice is accompanied by a decrease in metabolic rate and a decrement in internal body temperature.⁵⁶ The crude extract and all the three fractions (with the effect of n-butanol being the highest among the fractions) did have protective effects against temperature reduction in both models. Therefore, the antimalarial effect of the plant along with its antipyretic effect^{9,13} has a substantial effect with regard to prevention of body temperature decline during malaria infection. Moreover, malaria infection has also been associated with decreased food intake, disturbed metabolic function, and hypoglycemia.⁵⁷ Accordingly, both the crude extract as well as the fractions of the plant showed a moderate effect with regard to amelioration of body weight reduction. This activity might have resulted from the overall improvement in PCV, rectal temperature, and parasite clearance among treated mice. Nevertheless, the aqueous fraction did not prevent weight reduction significantly. This finding is in agreement with previous studies on aqueous fraction of other plants.^{29,58}

Therefore, according to this study, it is trustworthy to assume that the hydro-methanolic extract as well as the n-butanol and chloroform fractions possess a good antimalarial activity, justifying the previous in vitro studies as well as the ethnobotanical claims of *H. forskalei*. Although the plant showed a toxic effect to the aquatic lives⁵⁹, the lead compound responsible for the anti-malarial effect will be isolated from other toxic compounds upon bioactive guided fractionation of the extract. Despite the fact that the acute toxicity result of the plant reveals its safety in mice, it is most important to perform a well-designed preclinical study to find a lead compound with desired efficacy and safety for clinical study.

Conclusion

The study has revealed that the 80% methanolic extract of the leaf of *H. forskalei* has a promising schizontocidal and

curative potential during malaria infection. Besides, it has further shown that active principles are found to be more concentrated in the n-butanol and chloroform fractions. Thus, compounds ranging from semi-polar to non-polar are more likely to be responsible for the observed anti-malarial effect. Therefore, the experimental plant, *H. forskalei*, could be a potential source of new antimalarial drug with further bio-guided isolation of the active compound/s.

Abbreviations

H. forskalei, *Hypoestes forskalei*; *P. berghei*, *Plasmodium berghei*; 80ME, 80% methanol extract; PCV, packed cell volume; RBC, red blood cell; SEM, standard error of the mean; WHO, World Health Organization.

Data Sharing Statement

Data are all contained within the manuscript.

Ethics Approval and Consent to Participate

This study makes use of mice and the experimental protocol for the use of animals was in accordance with the national research council of the national academies (Institute for Laboratory Animal Research) and was approved by the Research and Ethics Committee of the department of Pharmacy of Wollo University with ethical approval number WU Phar/276/19, Dessie, Ethiopia.

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Author Contributions

All authors contributed to data analysis, drafting, or revising the article, have agreed on the journal to which the article will be submitted, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

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Disclosure

The authors report no conflicts of interest for this work.

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