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Antimalarial activity of hydromethanolic extract and its solvent fractions of *Vernonia amygdalina* leaves in mice infected with *Plasmodium berghei*

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Abstract

Background: *Vernonia amygdalina* Del. (Asteraceae) is reported to be traditionally used for the treatment of malaria. Based on folkloric repute of this plant in Ethiopian traditional medicine and crude extract-based ethnopharmacological studies conducted in few countries, this study was undertaken to evaluate the in vivo antimalarial activity of 80% methanol extract and its solvent fractions of the leaves of *V. amygdalina* in mice infected with *Plasmodium berghei*.

Methods: A 4-day suppressive test was conducted on mice infected with *P. berghei* to find out antimalarial effect of chloroform, butanol and aqueous fractions obtained from the 80% methanol crude extract. In all the activity tests, mice were randomly assigned in five groups (three tests and two controls) of six animals in each and received respective treatments. Data were analyzed using one way analysis of variance followed by Tukey's post hoc test for multiple comparisons.

Results: Acute oral toxicity test showed that all solvent fractions of the leaves of *V. amygdalina* revealed neither mortality nor overt signs of toxicity up to 2000 mg/kg. This study indicated that the percentage parasitemia suppression of 80% methanol extract was 32.47% (±2.65), 35.40% (±3.14) and 37.67% (±2.50) at 200, 400 and 600 mg/kg, respectively. All doses of the 80% methanol extract of *V. amygdalina* prolonged survival time and prevented weight loss and packed cell volume reduction in infected mice. All doses of chloroform and butanol fractions significantly suppressed parasitemia ($p < 0.05$), increased survival time ($p < 0.05$) compared to negative control and exhibited a significant reduction in rectal temperature ($p < 0.05$). All solvent fractions significantly prevented weight loss ($p < 0.05$) at all tested doses. The 80% methanol extract and chloroform and butanol fractions significantly ($p < 0.05$) prevented further reduction in rectal temperature of *P. berghei*-infected mice at all doses.

Conclusion: The results of this study indicated that 80% methanol extract and solvent fractions of the leaves of *V. amygdalina* demonstrated promising antimalarial activity. The study corroborated the folklore use of this plant for the treatment of malaria in ethnomedicine in Ethiopia.

Keywords

Antimalarial activity, 4-day suppressive test, *P. berghei*, solvent fractions, *V. amygdalina*

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Background

There were an estimated 216 million cases of malaria in 91 countries, an increase of 5 million cases over 2015. Nearly 90% of the cases in 2016 were in the World Health Organization (WHO) African Region (194,000), followed by the WHO South-East Asia Region (7%). Malarial deaths reached 445,000 in 2016, a similar number (446,000) to 2015. The WHO African Region continues to bear the brunt of the global burden of malaria. In 2015, 88% of global cases and 90% of global deaths occurred in the African Region.[1,2] In Ethiopia, about 55.7 million people (68% of the population) are at risk of malaria. Immunity against the disease in the majority of the Ethiopian population is relatively low due to the seasonal and unstable transmission and unlike large parts of sub-Saharan Africa; all age groups are at risk of infection. *Plasmodium falciparum* and *Plasmodium vivax* account for 65%–75% and 25%–35% malarial infections, respectively, while *Plasmodium ovale* and *Plasmodium malariae* occur rarely.[3]

WHO estimates that approximately 80% of the world's inhabitants rely on traditional or herbal medicines for their primary health care and medicinal plants have provided excellent leads for new drug development.[4,5] Medicinal plants have now become one of the hot spots for the search of new antimicrobial agents.[6–9] In the 21st century, there is alarmingly increasing antimicrobial resistance on the available agents with very slow development of new antimicrobials.[10,11] Hence, we are really facing a post-antimicrobial era where the present day agents become historical.[12,13] In the face of increasing problem by drug-resistant malaria parasite, there is still a need for intensification of research into medicinal plant claim to be effective in malaria management.[14] It is, therefore, mandatory to extensively search new agents based on the acclaimed traditional use of herbal medicines as one of the potential sources.

In Ethiopia, *Vernonia amygdalina* Del. (Asteraceae) has a folkloric repute for the treatment of malaria.[15] Crude ethanolic extracts of the leaves and root-bark of *V. amygdalina* suppressed parasitemia in mice by 67% and 54%, respectively, in a 4-day suppressive test.[16] Another study revealed suppressive effect of the crude ethanol extract of the plant in vivo in a dose-dependent manner with maximum activity observed at 1000 mg/kg with percentage inhibition of 82.3%.[17] Crude aqueous extract of the leaves of *V. amygdalina* also reduced parasitemia in mice by 73% when given intraperitoneally for 4 days.[18] Crude extracts of ethanol, petroleum ether, dichloromethane, ethyl acetate, acetone-water and isoamyl alcohol extracts of *V. amygdalina* showed antimalarial activity against *P. falciparum* in vitro.[19,20] All the aforementioned antimalarial tests were conducted in different countries and were crude extracts based. Therefore, this study has attempted to go one step forward to address the antimalarial activity of solvent fractions of the hydromethanolic extract.

Materials and methods

Plant material collection

The leaves of *V. amygdalina* were collected in January 2015 from Kombolcha area, Amhara Regional State, 376 km north-east of Addis Ababa, the capital of Ethiopia and brought to College of Natural and Computational Sciences (CNCS), Addis Ababa University (AAU). The plant material was authenticated by a botanist (Mr Melaku Wondafrash) at CNCS, and a voucher specimen was deposited at the National Herbarium of AAU with reference number TB001/2015.

Preparation of 80% methanol extract

The leaves of *V. amygdalina* were cleaned with tap water, air-dried under shade at room temperature for 2 weeks and ground into powder using a grinding mill at the Pharmacology Department of AAU. A total of 900 g powder was added into 600 mL 80% methanol (universal solvent) in a separate Erlenmeyer flask and was put on a mini-orbital shaker (Bibby Scientific Limited, Stone, Staffordshire, UK) at 120 r/min for 72 h. After 72 h, the extracts were filtered using gauze followed by Whatman No.1 filter paper (Wagtech international Ltd, RG19 4HZ Thatcham, Berkshire, England). The residues left after extraction were separately re-macerated twice using the same volumes of 80% methanol to exhaustively extract the plant material. The methanol part in the 80% methanol extract (80ME) was removed by evaporation under reduced pressure with a rotary evaporator (Buchi Rotavapor, Switzerland) at 80 r/min and 40°C to obtain the concentrated extract. Following deep freezing of the aqueous solvent in to ice, the extract was further concentrated to dryness by freeze drying using lyophilizer (Operon, Korea vacuum limited, Korea). Finally, the extraction process yielded a total of 128.5 g (14.23% w/w) of dried 80ME.

Preparation of solvent fractions

The 80ME was processed for further fractionation using chloroform, butanol and distilled water. In this case, 90 g of the 80ME was dissolved in 350 mL distilled water using a separatory funnel. The dissolved 80ME was partitioned with 3×240 mL chloroform. The filtrate was concentrated in rotary evaporator at 80 r/min and 40°C to obtain chloroform fraction (CF). The aqueous residue was further partitioned with 3×240 mL n-butanol, and the butanol filtrate was similarly concentrated to get butanol fraction (BF). The remaining aqueous residue was frozen in deep freezer overnight and then freeze-dried with the lyophilizer to obtain aqueous fraction (AF). The 80ME and the fractions were kept in airtight containers in a refrigerator at –20°C until use.[21,22] The BF and AF were dissolved with distilled water, whereas CF was dissolved in 2% Tween-80 for use in the tests. Fractionation of the 80ME yielded 26 g (28.9%), 20.29 g (22.54%) and 14 g (15.6%) of AF, CF and BF, respectively.

Experimental animals

Swiss albino mice aged 6–8 weeks and weighing 22–30 g were used for the experiment. The animals were obtained from animal center of Ethiopian Public Health Institute (EPHI), Addis Ababa, and interbred at the animal house of Department of Pharmacology, School of Medicine, AAU. Five to eight mice were housed in polyethylene cages having a metallic cover with woodchip bedding at ambient room temperature and exposed to a 12 h light/dark cycles with free access to standard pellet food and water. The mice were acclimatized to laboratory condition for 1 week prior to the experiment. Female mice were used for the acute oral toxicity test, whereas male mice were used for in vivo antiplasmodial activity of the leaves of *V. amygdalina* at the department of Pharmacology, School of Medicine, AAU. The use and handling of mice was in accordance with the guideline for the care and use of experimental animals.[23]

Acute oral toxicity test

Acute oral toxicity test of the 80ME and solvent fractions was performed on randomly selected 25 non-infected female mice following the Organization for Economic Corporation and Development (OECD) guideline.[24] The mice were fasted overnight and weighed before the test. A single loading dose of 2000 mg/kg of each extract was administered to single mouse with oral gavage. Then, any sign of overt toxicity and/or mortality were observed for 24 h with special emphasis to the first 4 h. As no death or over toxicity was observed within 24 h, additional four female mice were recruited and dosed similarly in each extract and followed for 14 days to assess delayed toxicity of the 80ME and solvent fractions. The mice were observed for any potential signs of acute toxicity such as loss of appetite, hair erection, lacrimation, tremors, convulsions, salivation and/or mortality.[25]

Parasite inoculation, grouping, and antimalarial activity testing

Chloroquine-sensitive *P. berghei* (ANKA strain) obtained from EPHI and maintained by subsequent passage of blood from infected mouse to a healthy one every 5 days was used for the experiment. The antiplasmodial activity of the 80ME was assessed following procedures used in earlier studies[16,18,26] in 30 male mice randomly divided into five groups of six mice in each. Each mouse was inoculated with 0.2 mL *P. berghei*-infected blood intraperitoneally. Blood from a donor mouse with a rising parasitemia of about 20%–30%[27,28] was used to infect test mice. Donor mouse was euthanized using halothane and blood was immediately collected through cardiac puncture in to a petri-dish containing 2% trisodium citrate as an anticoagulant. The blood was then diluted with physiological saline (0.9%)[29] in such a way that 1 mL blood contains 5×10^7 infected red blood cells (RBCs).[30]

For 80ME, mice in group I were treated with 0.2 mL distilled water (negative control), and those in groups II, III and IV were treated with doses of 200, 400 and 600 mg/kg extracts, respectively. The last group was assigned as positive control and received the standard drug, chloroquine (25 mg/kg) (obtained from Ethiopian Pharmaceutical Manufacturing, Addis Ababa, Ethiopia). The antimalarial activity of the solvent fractions was also assessed following the above-stated procedure. Male mice were organized into five groups of six per group. Mice in Group I (negative control) were treated with the vehicle (distilled water for AF and BF and 2% Tween-80 for CF) while those in groups II, III and IV were treated with 100, 200 and 400 mg/kg solvent fractions, respectively. Group V received 25 mg/kg chloroquine as a standard drug. Preparations were administered via oral route using mice oral gavage. The volume to be administered was calculated based on the body weight of an individual mouse, and 0.30 mL was the maximum volume administered.

Treatment of infected mice was started on day 0, 3 h after they had been inoculated with the parasite and continued for 3 days (from day 1 to day 3) with a 24-h time interval. At day 4 (i.e. 96 h post infection), thin blood film was made from each mouse, stained with 10% Giemsa, and percentage of parasite inhibition was determined by counting five fields of approximately 100–200 cells in each slide (numbers of infected and uninfected RBCs)[31–33] and taking the average count (the mean parasitemia) using the following formula[34]

$$\text{Percent parasitemia} = \frac{\text{Total number of parasitized } RBCs}{\text{Total number of } RBCs} \times 100$$

Parasitemia suppression of each fraction was compared with respect to the controls, and percent parasitemia suppression was calculated using the following formula[35]

$$\text{Percent suppression} = \frac{(\text{Parasitemia of negative control} - \text{parasitemia of treated group})}{\text{Mean parasitemia of negative control}} \times 100$$

The other important variables measured were body weight, body temperature, packed cell volume (PCV) and mean survival time (MST). Body weight was measured using sensitive digital balance (Mettler Toledo, Switzerland) before infection (day 0) and on day 4 to observe whether the extracts and fractions of the leaves of *V. amygdalina* prevented weight loss. Rectal temperature of each mouse was measured daily starting from day 0 (before infection) to day 4 by a digital thermometer to assess the effect of the 80ME and its fractions on body temperature. PCV was measured to determine the effectiveness of the crude extract and its solvent fractions in preventing hemolysis resulting from

increasing parasitemia using the modified Wintrobe's method to counteract PCV reduction.[36] Blood was taken from tail of the mouse with heparinized microhaematocrit tubes (filled to three-fourths of their height with blood), sealed with sealing clay, centrifuged for 5 min with 12,000 r/min, and PCV was determined by measuring the proportion of RBCs to plasma before inoculating the parasite (day 0) and after treatment (day 4) using the following formula[37]

$$\text{Packed cell volume } (\text{PCV}) = \frac{\text{Volume of erythrocytes in a given volume of blood}}{\text{Total blood volume}}$$

Determination of MST

Survival time was recorded to observe the effect of the crude extract and its solvent fractions on the survival of *P. berghei*-infected mice. The mice were fed ad libitum and observed for about 28 days. Any death that occurred during this period was noted for each mouse in the treatment and control groups to determine the MST[38]

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

Preliminary phytochemical screening of V. amygdalina

The 80ME and its solvent fractions were screened for the presence of phytochemical constituents (alkaloids, tannins, saponins, flavonoids, terpenoids, steroids, cardiac glycosides, anthraquinones and phenols) to relate the antimalarial activity with the availability of these phytoconstituents following standard procedures.[39–41]

Data analysis

Data were entered and analyzed using IBM Statistical Package for the Social Sciences (SPSS), version 20.0 software (Armonk, New York, United States). Results were expressed as mean plus or minus standard error of the mean (M ± SEM). Normality of data distribution was checked using Kolmogorov–Smirnov and Shapiro–Wilk test. One way analysis of variance (ANOVA) was performed following Tukey's honestly significant difference (HSD) post hoc test for multiple comparisons of differences in parasitemia, percent suppression, MST, changes in body weight, PCV and rectal temperature among groups. The analysis was performed at 95% confidence level and p value < 0.05 was considered as statistically significant.

Results

Acute oral toxicity of 80ME and its fractions

The 80ME and its solvent fractions of the leaves of *V. amygdalina* did not cause mortality in mice at the level of 2000 mg/kg. The 80ME and its fractions did not induce any sign of overt toxicity such as loss of appetite, hair erection, lacrimation, tremors, convulsions and salivation during the 14 days of observation.

The 80ME and its fractions on parasitemia suppression and survival time

The 80ME of *V. amygdalina* caused 32.47% (±2.65), 35.40% (±3.14) and 37.67% (±2.50) parasitemia suppression at 200, 400 and 600 mg/kg/day, respectively. An increase in doses of 80ME of the leaves of *V. amygdalina* was associated with increased parasite suppression. All doses of 80ME caused statistically significant ($p < 0.05$) parasite suppression and contributed to significantly higher survival time of the mice compared to the negative controls (Table 1). Besides, 21.68% (±5.41), 23.72% (±1.92) and 33.85% (±9.08) parasitemia suppression was observed in CF at 100, 200 and 400 mg/kg, respectively. The suppression effect of this fraction at all tested doses was significantly different ($p < 0.05$) as compared to the negative control (Table 1). Similarly, all the dose levels of BF produced significant ($p < 0.001$) parasitemia reduction as compared to the negative control (Table 1). However, only 400 mg/kg of AF demonstrated significant suppression ($p < 0.05$) as compared to the respective negative control. At the 400 mg/kg, CF produced chemosuppression of 33.85% (±9.08), which is higher than that of BF (26.88% ± 0.70%) and AF (9.75% ± 4.84%). CF and BF were capable of significantly ($p < 0.05$) increasing the survival time of mice at all tested doses compared to the negative control. However, AF significantly ($p < 0.05$) prolonged survival time only at 400 mg/kg. Besides, 400 mg/kg CF resulted in greater survival time of mice than that of BF and AF (Table 1).

The 80ME and its fractions on body temperature, body weight and PCV

All doses of the 80ME exhibited a significant reduction in body temperature of *P. berghei*-infected mice in a dose-dependent manner as compared to the negative control ($p < 0.05$). The 80ME at 400 and 600 mg/kg exhibited less reduction in body temperature as compared to their respective lower doses (200 mg/kg). All doses of 80ME significantly prevented weight loss as compared to negative controls ($p < 0.001$). The 80ME of the *V. amygdalina* leaves significantly prevented PCV reduction at all tested doses ($p < 0.001$) (Table 2). All the doses of CF and BF exhibited significant reduction in rectal temperature of *P. berghei*-infected mice in a dose-dependent manner ($p < 0.05$). Comparison among the different doses of CF revealed apparent difference in the

Table 1. Effect of 80ME and its solvent fractions of the leaves of *V. amygdalina* on parasitemia suppression and survival time of infected mice.

Category	Treatment	Dose (mg/kg)	% parasitemia	% suppression	MST (days)
Crude	NC	–	50.00 ± 0.58	0.00	6.50 ± 0.22
	80ME	200	33.77 ± 1.33	32.47 ± 2.65^{a***,b***}	9.50 ± 0.42^{a**,b***,c*}
	80ME	400	32.30 ± 1.57	35.40 ± 3.14^{a***,b***}	10.17 ± 0.79^{a***,b***}
	80ME	600	31.17 ± 1.25	37.67 ± 2.50^{a***,b***}	12.00 ± 0.58^{a***,b***}
	PC	25	0.00	100	29.67 ± 0.21
Solvent fractions	NC	–	47.13 ± 4.04	0.00	6.67 ± 0.33
	CF	100	36.88 ± 2.54	21.68 ± 5.41^{a*,b***}	8.5 ± 0.43^{a*,b***}
	CF	200	35.90 ± 0.89	23.72 ± 1.92^{a*,b***}	9.00 ± 0.58^{a**,b***}
	CF	400	31.15 ± 4.27	33.85 ± 9.08^{a***,b***}	9.83 ± 0.40^{a***,b***}
	PC	25	0.00	100	29.5 ± 0.34
	NC	–	49.81 ± 3.83	0.00	6.33 ± 0.49
	BF	100	41.60 ± 2.45	18.12 ± 4.82^{a***,b***}	7.83 ± 0.31^{a*,b***}
	BF	200	39.33 ± 0.31	21.03 ± 0.62^{a***,b***}	8.50 ± 0.0.23^{a*,b***}
	BF	400	36.55 ± 0.35	26.88 ± 0.70^{a***,b***}	9.17 ± 0.31^{a***,b***,d***}
	PC	25	0.00	100	29.33 ± 0.33
	NC	–	57.78 ± 3.09	0.00	6.00 ± 0.26
	AF	100	56.42 ± 2.89	5.60 ± 3.60^{b***}	6.00 ± 0.37^{b***,c*}
	AF	200	55.33 ± 0.83	6.72 ± 1.74^{b***}	6.50 ± 0.22^{b***}
	AF	400	52.55 ± 2.97	9.75 ± 4.84^{b***}	7.33 ± 0.21^{a*,b***}
	PC	25	0.00	100	29.5 ± 0.34

Data are expressed as mean ± SEM; n = 6; NC = negative control (0.2 mL tween-80/distilled water); PC: positive control (chloroquine); AF: aqueous fraction; BF: butanol fraction; CF: chloroform fraction; MST: mean survival time.

[a]Compared to negative control.

[b]Compared to positive control.

[c]Compared to 600 mg/kg (80ME) and 400 mg/kg (solvent fractions).

[d]Compared to chloroform.

*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

prevention of temperature decline between the 100 and 400 mg/kg doses ($p < 0.001$). BF at a dose of 400 mg/kg showed a significant activity in reducing rectal temperature compared to the negative control ($p < 0.001$). Chloroquine significantly prevented the decline in rectal temperature as compared to all doses of both fractions (Table 2). All doses of CF, BF and AF significantly ($p < 0.001$) prevented loss of body weight of mice as compared to the negative control (Table 2). It was shown that the CF and BF of *V. amygdalina* significantly ($p < 0.01$) prevented the PCV reduction at all dose levels compared to the negative control. However, the AF did not show statistically significant prevention of PCV reduction compared to the negative control (Table 2).

Preliminary phytochemical screening

Phytochemical screening of the 80ME of the leaves of *V. amygdalina* revealed the presence of alkaloids, tannins, saponins, flavonoids, cardiac glycosides, terpenoids, anthraquinones and phenols (Table 3). Regarding its solvent fractions, phytochemical screening of CF, BF and AF of the leaves of *V. amygdalina* revealed the presence of alkaloids and saponins. Besides, flavonoids are present in both CF and BF. Generally, the CF was found to have a higher number of secondary metabolites in type.

Discussion

Based on the acute toxicity study, the LD_{50} of 80ME and its solvent fractions of the leaves of *V. amygdalina* were found to be greater than 2000 mg/kg, indicating their wide safety margin. The present result is in line with the finding of Adiukwu et al.[42] who reported that *V. amygdalina* caused no clinical signs of toxicity at doses between 500 and 2000 mg/kg/day for 14 consecutive days and that of Njan et al.[18] who reported the absence of signs of overt toxicity or adverse toxicological effects at all tested doses. Amole et al.[43] also found no toxic effect of extracts of *V. amygdalina* in vivo on rats. Generally, if LD_{50} value of the test chemical is more than three times the minimum effective dose, the substance is considered to be a good candidate for further studies in vivo assays.[24,44,45] The LD_{50} has also been used for classification of chemicals. Based on WHO hazard classification system, the crude extract and its solvent fractions of the leaves of *V. amygdalina*, to which the LD_{50} was greater than 2000 mg/kg, are designated as "unlikely to be hazardous."[46] Therefore, the 80ME and its fractions of the plant are considered to be safe at the tested doses. The aforementioned descriptive toxicological studies also support this finding.

The 4-day suppressive test on *P. berghei*-infected mice is the standard test commonly used for antimalarial drugs screening,[47]

Table 2. Effect of 80ME and its solvent fractions on body temperature, body weight and PCV of *P. berghei*-infected mice.

Category	Treatment	Dose mg/kg)	Body Temperature (°C)			Body Weight (g)			Packed cell volume		
			D0	D4	% change	D0	D4	% change	D0	D4	% change
Crude	NC	–	36.3 ± 0.1	34.1 ± 0.2	−3.4 ± 0.1	24.2 ± 0.8	21.0 ± 0.4	−15.3 ± 2.8	76.9 ± 0.9	52.5 ± 1.18	−46.9 ± 4.2
	80ME	200	36.7 ± 0.2	34.8 ± 0.1	−2.1 ± 0.6^{a*,b***,d*}	24.9 ± 1.2	26.2 ± 0.1	5.3 ± 0.6^{a***}	75.3 ± 1.7	64.5 ± 1.24	−16.9 ± 3.2^{a***}
	80ME	400	36.7 ± 0.2	35.1 ± 0.3	−1.6 ± 0.1^{a***,b*}	22.7 ± 0.6	24.4 ± 0.6	6.9 ± 1.0^{a***}	77.8 ± 2.3	68.7 ± 2.37	−13.3 ± 1.3^{a***}
	80ME	600	36.6 ± 0.2	35.2 ± 0.3	−1.0 ± 0.5^{a***}	22.8 ± 0.3	24.7 ± 0.4	7.7 ± 1.0^{a***}	76.5 ± 1.8	67.8 ± 2.17	−13.1 ± 2.7^{a***}
	PC	25	36.5 ± 0.4	36.1 ± 0.4	−0.3 ± 0.1	25.5 ± 0.6	28.7 ± 0.3	10.8 ± 1.7	85.3 ± 0.8	78.6 ± 0.9	−7.5 ± 0.5
Solvent fractions	NC	–	36.0 ± 0.2	34.9 ± 0.2	−3.3 ± 0.1	25.3 ± 1.2	23.2 ± 0.9	−9.4 ± 2.3	81.4 ± 1.4	54.5 ± 3.7	−53.2 ± 12.3
	CF	100	37.1 ± 0.3	36.1 ± 0.3	−2.7 ± 0.1^{a**,b***,c**}	25.0 ± 0.7	25.8 ± 0.7	3.0 ± 1.1^{a***,b*}	77.6 ± 1.8	64.5 ± 1.5	−20.4 ± 2.0^{a**}
	CF	200	35.8 ± 0.3	35.0 ± 0.3	−2.3 ± 0.1 ^{a***,b***}	28.0 ± 0.3	29.0 ± 0.5	3.5 ± 1.0^{a***,b*}	75.4 ± 1.4	63.0 ± 1.4	−19.8 ± 1.7^{a**}
	CF	400	36.1 ± 0.3	35.4 ± 0.3	−1.9 ± 0.2^{a***,b***}	26.7 ± 0.3	27.5 ± 0.2	4.1 ± 0.7^{a***}	75.3 ± 1.7	64.5 ± 1.2	−16.9 ± 3.2^{a***}
	PC	25	36.4 ± 0.3	36.2 ± 0.3	−0.5 ± 0.1	29.1 ± 0.7	32.3 ± 0.4	9.8 ± 2.1	75.2 ± 3.5	67.8 ± 3.0	−10.8 ± 1.6
	NC	–	36.0 ± 0.2	34.9 ± 0.2	−3.3 ± 0.1	25.3 ± 1.2	23.2 ± 0.9	−9.4 ± 2.3	81.4 ± 1.4	54.5 ± 3.7	−53.2 ± 12.3
	BF	100	35.9 ± 0.3	34.9 ± 0.3	−2.9 ± 0.3^{a***,b***,c**,d***}	26.3 ± 0.3	26.9 ± 0.3	2.1 ± 0.3^{a***,b**}	81.7 ± 1.0	67.2 ± 2.5	−22.2 ± 3.5^{a**}
	BF	200	36.0 ± 0.1	35.1 ± 0.1	−2.5 ± 0.6^{a***,b***,d***}	24.4 ± 0.7	24.9 ± 0.6	2.2 ± 0.4^{a***,b**}	80.5 ± 1.3	66.9 ± 2.8	−21.1 ± 3.7^{a**}
	BF	400	35.7 ± 0.1	35.0 ± 0.1	−2.0 ± 0^{a***,b***}	27.9 ± 1.3	28.7 ± 1.3	2.5 ± 0.1^{a***,b**}	78.6 ± 3.7	66.5 ± 2.8	−18.1 ± 1.4^{a**}
	PC	25	35.9 ± 0.3	36.4 ± 0.3	−0.3 ± 0.1	29.3 ± 1.8	32.5 ± 1.5	10.1 ± 0.2	75.8 ± 3.7	68.8 ± 3.4	−10.2 ± 1.4
	NC	–	35.9 ± 0.2	34.7 ± 0.2	−3.6 ± 0.1	27.9 ± 1.3	23.6 ± 1.1	−18.2 ± 1.5	80.4 ± 2.5	54.6 ± 4.1	−50.8 ± 10.2
	AF	100	35.7 ± 0.4	34.6 ± 0.4	−3.4 ± 0^{b***}	24.3 ± 1.5	24.7 ± 1.5	1.6 ± 0.3^{a***,b***}	77.6 ± 1.8	56.3 ± 1.7	−38.0 ± 3.2^{b*}
	AF	200	35.5 ± 0.4	34.3 ± 0.3	−3.3 ± 0^{b*}	26.1 ± 1.3	26.6 ± 1.3	1.9 ± 0.4^{a***,b***}	75.4 ± 1.4	55.7 ± 1.2	−35.6 ± 2.0^{b*}
	AF	400	35.5 ± 0.3	34.4 ± 0.3	−3.3 ± 0^{b***}	26.9 ± 1.4	27.5 ± 1.4	2.1 ± 0.9^{a***,b***}	74.8 ± 3.6	57.2 ± 2.8	−31.1 ± 4.6^{b*}
	PC	25	36.1 ± 0.2	35.9 ± 0.2	−0.5 ± 0.1	27.2 ± 1.4	30.5 ± 1.3	11.0 ± 0.8	75.2 ± 3.5	68.1 ± 3.2	−10.4 ± 1.8

Data are expressed as mean ± SEM; n = 6; NC: negative control; PC: positive control; AF: aqueous fraction; BF: butanol fraction; CF: chloroform fraction.
[a]Compared to negative control.
[b]Compared to positive control (chloroquine).
[c]Compared to 200 mg/kg.
[d]Compared to 600 mg/kg (80ME) 400 mg/kg (fractions).
*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; D0, at day 0; D4, at day 4.

Table 3. Phytochemical screening of leaves of 80ME and its fractions of *V. amygdalina*.

Phytochemical constituents	Crude	Solvent fractions			Controls (solvents)	
	80ME	AF	BF	CF	DW	Tween 80
Alkaloids	+	+	+	+	−	−
Tannins	+	−	−	+	−	−
Saponins	+	+	+	+	−	−
Flavonoids	+	−	+	+	−	−
Terpenoids	+	−	−	+	−	−
Steroids	−	−	−	−	−	−
Cardiac Glycosides	+	−	−	+	−	−
Anthraquinones	+	−	−	−	−	−
Phenols	+	+	−	+	−	−

+ indicates the presence of phytochemical constituent; − indicates the absence of phytochemical constituent.
AF: aqueous fraction; BF: butanol fraction; CF: chloroform fraction; 80ME: 80% methanol extract; DW: distilled water.

and determination of percent suppression of parasitemia is the most reliable parameter. In vivo antiplasmodial activity can be classified as moderate, good and very good if an extract displays percent parasite suppression equal to or greater than 50% at a dose of 500, 250 and 100 mg/kg/day, respectively.[48,49] Based on this classification, 80ME and its CF of *V. amygdalina* leaves exhibited relatively smaller antiplasmodial activity as they had exhibited parasitemia suppression of 37.67% at a dose of 600 mg/kg and 33.85% at a dose of 400 mg/kg, respectively. The higher antimalarial activity reported in previous studies authored by Abosi and Raseroka[16] and Omoregie et al.[17] compared with the present finding might be related to the difference in geographical distribution, time of collection (seasonal variation), stage of development and/or extraction processes which can independently or in concert affect the quality and quantity of phytochemical constituents. It is crystal clear that several exogenous (altitude, day length, collection season, soil condition and allelopathy) and indegenous factors (morphological variety and biochemical diversity) and postcultivation factors possibly affect the quality and quantity of bioactive secondary metabolites. No matter how low the level of parastemia suppression is, standardized and published in vivo antimalarial assay is used in this case. Interestingly, the presence of significant activity on body weight, body temperature, PCV and MST, which are also important parameters of an antimalarial assay, should be considered while treating malaria.

The overall antimalarial activity of the 80ME and its fractions in this investigation might be due to the presence of bioactive secondary metabolites contained in the plant including terpenoids, alkaloids, phenols, flavonoids and tannins. Sesquiterpene lactones, class of naturally occurring plant terpenoids, which are most prevalent in the family of Asteraceae where this plant belongs to,[50] are known for their antimalarial activity.[20,51,52] Terpenoids also play an ecological role in the interaction of plants with their environment and have been shown to have a broad range of biological activities such as antibiotic, cytotoxic, antimalarial, antifeedant, insecticidal, molluscicidal and herbicidal properties. For example, the anticancer drug, taxol and the antimalarial drug artemisinin are widely known terpene-based drugs.[51] Alkaloids exert their antimalarial effect by disrupting the parasite ability of detoxifying heme into non-toxic malaria pigment, which by doing so exposes the parasite to toxic heme compounds.[53] Several phenolic compounds, flavonoids and tannins are also reported to have antiplasmodial activities.[54,55]

Results of this investigation on 80ME of the leaves of *V. amygdalina* revealed reduced parasitemia suppression as compared to that of Challand and Willcox[56] and Iwalokun. The antiplasmodial effect exhibited by the 80ME of *V. amygdalina* was comparable to the results of a previous study[57] conducted on other plants. The ethanol and aqueous stem bark extracts of *Pseudocedrela kotschyi* demonstrated a parasitemia suppression of 39.43% and 28.36%, respectively, at dose of 200 mg/kg, and methanol and water extracts of *Aloe debrana* produced parasitemia suppression of 30.21% and 23.53%, respectively, at a dose of 200 mg/kg.

With regard to other parameters of in vivo antimaraial bioassays, MST is important to evaluate the antimalarial activity of plant extracts.[58] Except for AF, all doses of the 80ME, BF and CF of *V. amygdalina* prolonged the survival time of mice which could be due to suppression of parasitemia and reduction of the overall pathologic effect of the parasite on the mice under study.[33] The different secondary metabolites present in the plant might have played a role in prolonging the survival time of the infected mice. The higher survival time in test groups has also substantiated the acute toxicity study with an extended follow-up period of 28 days.

A decrease in the metabolic rate of parasitized mice occurs before their death as a result of a decrease in internal body temperature.[59] On the contrary, active compounds are expected to prevent the rapid dropping of rectal temperature. At all dose levels, the crude extracts, CF and BF had protective effects against temperature reduction, and this reflects the presence of active constituents in good concentration.

Weight decrease in malaria-infected mice has been associated with decreased food intake, disturbed metabolic function and hypoglycemia.[33,60] Antimalarial agents obtained from plants are expected to prevent body weight loss in infected mice due to overall pathologic effect caused by the parasite. In this study, except for AF, all doses of the 80ME and CF and BF of *V. amygdalina* leaves significantly prevented weight loss in a dose-dependent manner due to their parasitemia suppressing activity. This indicates that the extracts had suppressed *P. berghei* and thereby reduced anemia and the overall pathologic effect of the parasite on the test groups.[33]

P. berghei-infected mice suffer from hemolytic anemia because of RBC destruction, due to either parasite multipli-

cation or spleen reticuloendothelial cell action as the presence of many abnormal RBC stimulates the spleen to produce many phagocytes.[61] This necessitates PCV analysis to evaluate the effectiveness of test materials in preventing hemolysis. In this study, it was noted that 80ME, CF and BF of *V. amygdalina* significantly prevented reduction in PCV at all dose levels as compared to negative control. This could be due to antiplasmodial effect of the crude extracts and fractions and as a result of sustaining the availability of new RBCs produced in the bone marrow. Failure of the AF to reverse PCV reduction could probably be related to the presence of higher concentration of saponins which are known to have strong hemolytic effects.[62]

Limitation of the study

This study tied to address the parasitemia-suppressive potential of this medicinal plant along with visible effects on other important parameters like PCV, MST, body weight and rectal temperature. However, the bioactive constituents of the plant material are yet to be purified and investigated for using them as antimalarial agents in modern medicine. Besides, this study could not evaluate the prophylactic activity of this medicinal plant.

Conclusion

The result of the in vivo acute toxicity study of 80ME and its solvent factions of the leaves of *V. amygdalina* in mice revealed no signs of toxicity indicating a good safety profile of the plant material. The results indicated that 80ME and its fractions of *V. amygdalina* possessed promising antimalarial activity. CF was found to be relatively the most active fraction which might contain potential lead compound(s) for the development of a new drug for the treatment of malaria. The antimalarial action of 80ME and solvent fractions of *V. amygdalina* has been attributed to the presence of bioactive secondary metabolites in the plant material. This supports the acclaimed traditional use of this plant by local communities in Ethiopia to treat malaria. More studies are needed to isolate and characterize active antiplasmodial constituents in the solvent fractions of this plant.

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Animal welfare

This study followed international, national, and/or institutional guidelines for humane animal treatment and complied with relevant legislation. The mice were handled in accordance with national guidelines for handling laboratory animals. The ARRIVE guideline was also strictly followed.

Authors' contributions

T.B. drafted the proposal and M.G., A.A. and G.Y. reviewed it. T.B. and M.S. conducted the experiment, analyzed and interpreted the data. All authors participated in the write-up of the manuscript. All authors have read and approved the final manuscript.

Availability of data and materials

Specimen of the tested antimalarial plant was deposited at the National Herbarium of the Addis Ababa University with voucher number TB001. The data are available in public library of Addis Ababa University in a form of institutional repository.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Ethical approval

The research proposal was reviewed and approved by the Institutional Review Board of the Department of Pharmacology, School of Medicine, College of Health Sciences, Addis Ababa University with approval number 02/04/2014.

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