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In-vivo anti-malarial activity of crude extract and solvent fractions of the roots of *Clematis simensis* Fresen.(Ranunculaceae) in *Plasmodium berghei* infected mice

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Abstract
Background*: With the increasing resistance to currently available anti-malarial drugs, the search for newer agents has become imperative. Clematis simensis is known for its anti-malarial use in the Ethiopian folklore medicine but no study has validated this claim so far. Therefore, the present study was intended to evaluate the anti-malarial activities of the crude extract and solvent fractions of the root part.*
Methods*: The in vivo anti-malarial activities of hydro alcoholic crude extract, aqueous, chloroform and n-hexane fractions of C. simensis roots (at 100, 200,400 and 600 mg/kg) were evaluated using a chloroquine sensitive rodent malaria parasite, P. berghei in Swiss Albino mice. The four-day suppressive and Rane's test were followed with the outcome parameters being parasitemia level, survival time, body weight, rectal temperature, and packed cell volume.*
Results*: When compared to the vehicle, the highest anti-malarial activity was observed at the 600mg/kg dose of the crude extract ($p<0.001$) and the 400mg/kg dose of the chloroform fraction ($p<0.01$) in the 4-day suppressive model. In the Rane's test as well, the chloroform fraction exhibited significant anti-malarial activity at the 400mg/kg ($p<0.05$) and 600mg/kg ($p<0.001$) doses.*
While the crude extract at 600mg/kg prevented only the decrease in rectal temperature, the 400mg/kg dose of chloroform fraction, in addition, significantly increased the survival time in the 4 days suppressive test. In the Rane's test, the 600mg/kg dose of chloroform fraction increased the survival time prevented a decrease in rectal temperature as well as packed cell volume significantly.
The crude extract did not reveal any obvious acute oral toxicity at 2000 mg/kg and phytochemical screening demonstrated the probable presence of alkaloid, flavonoid, terpenoid, and phenolic compounds.
Conclusion: *The findings of the present study revealed that the hydro alcoholic crude extract and chloroform fraction of C. simensis roots have significant anti-malarial activities and hence supported the use of the plant to manage malaria in the Ethiopian folklore medicine. Further work is necessary to make use of the plant in isolating, identifying and characterizing the active principles.*
Keywords*: C. simensis, herbal medicine, extract, in-vivo, malaria, P. berghei.*

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I. Introduction

Although malaria is a preventable and curable disease, it remains to be a major public health problem. It is one of the top three killer infectious diseases globally along with HIV/AIDS and tuberculosis [1]. In the year 2015, the number of malaria deaths globally was 438,000 and the Sub-Saharan Africa region was home to 90% of the deaths [2]. It has been estimated to cost the continent Africa more than US$12 billion every year in lost Gross Domestic Product [3]. As a result of successful strategies, mortality rates were decreased globally by 47% between 2000 and 2013 [4].

In Ethiopia, nearly 60% of the population lives in malarious areas [5] and 4–5 million cases of malaria and 70,000 related deaths have been reported annually [6]. The disease is unstable and seasonal [7], with the high transmission season overlapping with the cultivation months. Hence it affects agricultural production [8, 9]. It also accounts for 30% of the overall Disability Adjusted Life Years lost in the nation [10], making it a significant hurdle to social and economic growth [11].

Reducing the burden of malaria has implications to several of the sustainable development goals in the Sub-Saharan Africa [12]. The World Health Organization (WHO) envisions reducing the incidence and mortality rates at least by 90% by 2030 [13]. Ethiopia aims to achieve zero deaths due to malaria in areas with malaria transmission by 2020 [14]. However, development and spread of mosquito resistance to insecticides as well as resistance to drugs including artemisinins are the main challenges to achieve these goals [2]. As a result, the search for new agents has become imperative, with traditionally used medicinal plants being an important target.

Nearly one-third to one-half of pharmaceutical drugs was originally derived from plants [15] and there is a growing public interest in natural therapies globally [16]. The WHO supports the use of medicinal plants provided they are proven to be efficacious and safe [17]. However, herbs have been shown to have a wide range of undesirable or adverse reactions some of which are life-threatening [16]. Thus, the safety of herbal medicines has become a major concern to both national health authorities and the general public [18].

The use of traditional medicines to treat malaria has become a source of the two main modern anti-malarial drugs, artemisinins and quinine derivatives. Over 1200 plant species from 160 families are used to treat malaria and fever. On average, a fifth of patients use traditional herbal remedies for malaria in endemic countries [19].

The family Ranunculaceae comprises 59 genera and the genus *Clematis* has about 355 species in the world which are mostly perennial woody vines [20, 21]. Twenty six species of the genus *Clematis* have been traditionally used in various systems of medicine for the treatment of ailments such as nervous disorders, syphilis, malaria, dysentery, rheumatism, asthma, and as analgesic, anti-inflammatory, diuretic, antitumor, antibacterial and anticancer [22]. Chemical and pharmacological studies have shown that triterpenoid saponins are the main components in the genus *clematis* having a wide spectrum of biological effects [23] such as analgesic, antifungal, antitumor, cytotoxic and cyclooxygenase-2 inhibition [20].

C. simensis, which has synonyms. *Orientalis* (Fresen.) O. Ktze. var. *Simensis* and *C.altissima*, Hutch [24], is known as "Azoareg" in Amharic [25]. It is a tall climbing shrubby plant, up to ≥ 20m [24]. It has been used through grinding and giving a finger tip nasally for malaria [26], root/twig-homogenization orally for malaria, cough and liver disease treatment [27]. The plant is shown to possess significant anti-inflammatory and anti-nociceptive effects [28].

Due to the increasing drug resistance, novel drugs are urgently needed. As experience shows traditionally used medicinal plants are potential areas of research for introducing alternative agents. The present study was therefore designed to evaluate the efficacy as well as acute oral toxicity of *C. simensis* used as malaria treatment in the Ethiopian folk medicine.

II. Methods

Plant materials

The fresh roots of *Clematis simensis* Fresen were collected around Gondar town, 738km North West of Addis Ababa during December 2016. The plant specimen was identified and authenticated by Department of Biology, College of Natural and Computational Sciences, University of Gondar and a Voucher specimen (MA001) was deposited at their herbarium for referencing**.**

Experimental animals and parasite

Swiss albino mice of either sex (age 8-9 weeks and weight of 23-28 g) were obtained from animal house at Department of Pharmacology, University of Gondar as well as from the Ethiopian Health and Nutrition Research Institute (EHNRI). They were maintained under standard conditions (temperature of 22 ± 3°C, relative humidity of 50-60% and 12 h light/12h dark cycle) with pellets and water *ad libitum* and acclimatized for one week to the experimental environment. The care and handling of animals were according to standard guide lines [29, 30].

Chloroquine sensitive strain of rodent plasmodium, *P.berghei* (ANKA), was obtained from EHNRI and maintained by serial passage of blood from infected mice to non-infected ones on weekly basis [31].

Extraction procedures for crude extract

The collected plant roots were cleaned with tap water and air dried at room temperature under shade after slicing it into small pieces to facilitate homogenous drying. A total of 1.9kg of dried roots was extracted by maceration (100g of dried roots in 600ml of 80% methanol) for 72 hrs [32]. The extraction process was

facilitated by using an orbital shaker at 120 rpm. The mixture was first filtered using sieve and then with Whatman filters paper (No.3, 15cm size with retention down to 0.1µm in liquids). The residue was re-macerated for another 72 hrs twice and filtered. The combined filtrates were dried by rotary evaporator (model-RE200, UK) under reduced pressure at a temperature of 40°C. The remaining aqueous part was further dried using a lyophilizer (Ningbo Scientz Biotechnology, China). After drying, a total yield of dry extract was determined and kept at −20°C until use.

Fractionation

The successive solvent-solvent extraction procedure was followed for the fractionation of the plant sample. The crude 80% methanolic extract of *C. simensis* (151g) was suspended in 450 ml of distilled water in separatory funnels and this was further diluted with 450 ml of n-hexane. After gently shaking for 5minutes, the extract was allowed to settle for 25 minutes to be separated into two distinct layers according to their density. The upper n-hexane layer was collected and the same procedure was repeated three times until colourless n-hexane layer was obtained. After the collection of n-hexane fraction of the extract, it was fractionated with 450ml chloroform three times as in explained before and the bottom chloroform layer was collected leaving the aqueous fraction at the top. The n-hexane and chloroform fractions were dried in an oven of 40^{o}C, and the aqueous fraction was further dried using a lyophilizer (Ningbo Scientz Biotechnology, China). Furthermore, the percentage yield for n-hexane, chloroform and aqueous fractions of the plant was determined. These dried fractions were then transferred into separate vials and stored at −20°C until use [33].

Phytochemical screening

A preliminary phytochemical study was performed on crude extract of the root using standard procedures [34, 35].

Acute toxicity testing

After being fasted for 3hrs for food but not water, five female Swiss albino mice weighing 25-27g were taken. The first mouse was given a single dose of 2 g/kg crude extract using oral gavage and then food was withheld for further 2 hours. The mouse was observed for 24hrs and two additional mice were dosed similarly and observed for any signs of toxicity daily for 14 days for gross changes such as loss of appetite, hair erection, lacrimation, tremors, convulsions, salivation, diarrhoea, weight change and other signs of overt toxicity [30].

Parasite inoculation

Donor mice of 20 and 25% parasitemia level were sacrificed by head blow and blood was collected by severing the jugular vein into a tube containing 0.4% tri-sodium citrate. This was diluted with physiological saline (0.9%) based on parasitemia level of the donor mice in such a way that 1 ml blood contains 5 × 107 infected erythrocytes. Then each mouse was inoculated intraperitoneally with 0.2ml of blood suspension containing about 1×107 *P. berghei* parasitized erythrocytes [36].

Dosing and grouping of animals

For each model, animals were randomly assigned to five groups each consisting of five mice. Group I (negative control) for the crude extract and aqueous fraction were treated with the distilled water while that of chloroform and hexane fractions with 7% Tween-80 suspension at 10ml/kg and group II (positive control) of the crude and the fractions were administered chloroquine 25mg/kg orally [31].

The groups III, IV, V and VI (for crude extract only) were orally administered with crude extract and solvent fractions of 100, 200, and 400mg/kg doses. For the crude extract, an additional 600mg/kg dosing was added because the lower doses were showing insignificant anti-malarial activity. All the extracts and the drug were given through intragastric route by using standard intragastric tube to ensure safe ingestion of the extracts and the drug.

The 4-days suppressive test

Evaluation of suppressive effect (schizontocidal activity) of the crude extract and solvent fractions on early infection against chloroquine sensitive *P. berghei* infected mice was done by randomly grouping the mice into five groups of five mice and inoculating with parasite as described above [31].

Groups III, IV, V and VI were orally administered with the crude extract and solvent fractions at doses of 100, 200, 400 and 600 (for crude extract only) mg/kg body weight, group I was administered orally with distilled water, while group II was administered orally with chloroquine 25 mg/kg body weight for four consecutive days (D0-D3). All treatments were started 3 h post- infection, then % parasite inhibition, parasitemia, weight change, PCV and survival times were determined.

Rane's test-curative test

The curative test was undertaken with chloroform solvent fraction which showed the highest parasitemia suppression in the 4-day suppressive test. The mice were injected intraperitoneally with standard inoculum of 1x 107 *P. berghei* infected erythrocytes on the first day (day 0). Seventy-two hours later, the mice were divided into five groups of five mice per group as described earlier [37] and treated accordingly. The treatments were continued once daily for 5 days starting from day 3 through day 7. On days 6, 7 and 8, Giemsa stained thin blood films were prepared from the tail of each mouse, then parasitemia level and survival time of the fraction were determined with respect to the control groups [38].

For the 4-day suppressive test, body weight of each mouse was measured before infection (day 0) and on (day-4) using a sensitive digital weighing balance (E11140, Switzerland) for curative treatment before infection (day0) and (day5). Likewise, rectal temperature was also measured with a digital thermometer before infection and after treatment for both test types [36].

Peripheral smear test for parasitemia

After four days of treatment, on the 5^{th}- day thin blood smears were made from the tail of each mouse [39]. The blood films were fixed with methanol, stained with 10 % Giemsa at pH 7.2 for 10 min, then the stained slides were washed gently using distilled water and air dried at room temperature. Parasitemia were determined under Olympus microscope (CX21FS1, China) with an oil immersion nose piece of 100 × magnifications by counting 5-fields of ~ 200 erythrocytes per field. The difference between the mean values of the control group (negative control taken as 100%) and those of the experimental groups were calculated and expressed as percent parasitemia reduction or activity [31, 40].

Activity=100− (Mean parasitemia treated /Mean parasitemia of control) ∗100

% Parasitemia = (No. of parasitized RBC /Total no. of RBC counted) ∗100

Packed cell volume measurement (PCV)

PCV is a measure of the proportion of RBCs to plasma and measured before inoculating the parasite and after treatment with the extract. PCV measurement was done to predict the effectiveness of the test extract and fractions in preventing haemolysis due to malaria parasites. Blood was collected by heparinized capillary tubes from the tails of each mouse. The capillary tubes were filled with each mouse's blood up to 75% of their volume and sealed at the heparin end with sealing clay. The tubes were then placed in a hematocrit centrifuge (Hettich Hematokrit-210, Germany) with the sealed end outwards and centrifuged for 5 min at 10,000 rpm. The tubes were then taken out of the centrifuge and PCV was determined using a standard Micro-Hematocrit Reader (Hawksley and Sons, England) [41].

PCV = (Volume of erythrocytes in a given volume of blood /Total blood volume)

Determination of mean survival time (MST)

Mortality was monitored daily and the number of days from the time of inoculation of the parasite up to death was recorded for each mouse in the treatment and control groups throughout the follow up period [42].

MST= (Sum of survival days of all mice in a group/Total number of mice in that group)

Data analysis

Results of the study were presented as mean ± SEM. Data were analyzed using SPSS version 20. Statistical significance was determined by One-way ANOVA coupled to Tukey's HSD technique to compare result between doses and among treatment and control groups. For all the data obtained, the result was considered significant at 95% confidence level and P-value < 0.05.

Ethical clearance

Animal handling was according to the standard guide lines for Care and Use of Laboratory animals [29, 30]. Furthermore, procedures were approved by the Institutional Review Board of the University of Gondar.

III. Results

Extract yield

The 80% methanolic crude extract of *C. simensis* was deep yellow semisolid mass at room temperature and the percentage yields for the crude extract, n-hexane, chloroform and aqueous fractions of the plant were 8.74%, 0.23%, 1.4% and 98.36% respectively.

Phytochemical screening

The preliminary phytochemical screening of 80% methanolic crude extract of *C. simensis* roots for the probable presence of different secondary metabolites is summarized in Table1.

Table 1 Phytochemical constituents of 80% methanolic crude extract of *C. simensis*

Phytochemical	Test type	Appearance when positive	Result
Alkaloids	Wagner's and Mayer's tests	Reddish brown and White creamy ppt.	+
Tannins	Braymer's test	Blue/Greenish colour	-
Saponins	Foam test	Foam	-
Flavonoids	Alkaline reagent test	Colour less	+
Terpenoids	H2SO4(3ml)+Chloroform(2ml)	Reddish brown	+
Steroids	H2SO4(10ml)+Chloroform(10ml)	Brown(bottom),colour less (upper)	-
Phenols	FeCl3	Bluish black	+
Glycosides	Keller-Killani test	Brown ring on interface	-
Anthraquinones	Chloroform + NaOH	Red colour	-

Note: (+) indicates presence; (-) indicates absence; ppt.-precipitate

Acute toxicity

The *in vivo* oral acute toxicity study indicated that the tested mice suffered neither mortality nor any other visible signs of toxicity like lacrimation, hair erection, and reduction in their motor and feeding activities for 14 days up at 2g/kg dose of the crude extract.

Effect of the crude extract on parasitemia and survival time in the 4-day suppressive test

As shown in Table2, the crude extract of *C. simensis* has shown a dose dependent antiplasmodial activity but it was significant only at the 600mg/kg dose. This dose demonstrated a 53.54% level of parasite suppression compared to the vehicle. This was found to be significant ($p < 0.001$) when compared to the vehicle, 100mg/kg as well as 200mg/kg doses.

Table 2. Effect of *C. simensis* crude extract on parasitemia and survival time in 4 days suppressive test

Extract	Treatment (mg/kg)	% Parasitemia	% Suppression	Survival time (day)
Crude	CS 100	57.6±5.21	-	6.0±0.32
	CS 200	50.2±5.38	11.65	6.6±0.40
	CS 400	44.6±3.4	21.5	$9.0 \pm 0.55^{a}{}_{1}{}^{b}{}_{1}$
	CS 600	$26.4 \pm 2.15^{a}{}_{3}{}^{b}{}_{3}{}^{c}{}_{3}{}^{d}{}_{1}$	53.54	$10.2 \pm 1.07^{a}{}_{3}{}^{b}{}_{3}{}^{c}{}_{2}$
	DW 10	56.82±2.2	0	6.0±0.45
	CQ 25	$0.00 \pm 0.00^{\infty}{}_{3}$	100	$30 \pm 0.00^{\infty}{}_{3}$

Results are expressed as mean ±SEM, n=5; a = compared to negative control (DW=Distilled Water); b= compared to100mg/kg; c= compared to 200mg/kg; 1= $p < 0.05$; 2 = $p < 0.01$; 3= $p < 0.001$; ∞ = all treatments; CS = *Clematis simensis;* CQ=Chloroquine

The survival time was also highest at the 600mg/kg dose of the crude extract which was significant compared to the vehicle ($p < 0.001$), 100mg/kg ($p < 0.001$) and 200mg/kg ($p < 0.01$) extract doses.

Effect of crude extract on body weight, rectal temperature and PCV measurements in 4-days suppressive test

Treatment with the crude extract, at all dose levels, did not increase body weight at completion of treatment in contrary to chloroquine. However the decrease in body weight was the smallest (0.64±0.11), though not significantly different, for the group treated with the crude extract at 600mg/kg dose. Table3. The decrease in rectal temperature at completion of treatment was small and significant ($p<0.05$) for both the 400mg/kg as well as 600mg/kg treated groups when compared to vehicle treatment. Regarding PCV, it has decreased for all doses and was the smallest at the 600mg/kg dose. There was so significant difference among the extract treatment groups compared to the negative control. The standard drug, however, demonstrated a significant activity in body weight increment ($p<0.05$), in preventing rectal temperature fall ($p<0.001$) and preventing PCV reduction ($p<0.01$) compared to the negative control.

Table 3. Effect of *C. simensis* crude extract on body weight, rectal temperature and PCV measurements in 4-days suppressive test

Treatment (mg/kg)	Body weight(g)			Rectal temperature(°C)			Packed cell volume (PCV)		
	B. Wt at D0	B. Wt at D4	% change	T at D0	T at D4	% change	PCV at D0	PCV at D4	% change
CS100	25.9±0.9	24.7±0.56	-1.25±0.79	36±0.20	35.3±0.2	-0.68±0.2	60.8±3.2	56.8±2.9	-4±0.78
CS200	24.9±0.8	23.5±1.2	-1.4±0.55	36.3±0.2	35.4±0.2	-0.88±0.15	60.6±2.7	56.8±2.6	-3.8±0.74
CS400	26.8 ±0.8	25.4±0.6	-1.37±1.37	36.7±0.2	36.1±0.1	$-0.64\pm0.09^{a}{}_{1}$	61.0 ±3.4	57.6 ±3.3	-3.4 ±0.51
CS600	26.4±0.5	25.7±0.4	-0.64±0.11	36.9±0.2	36.2±0.2	$-0.9\pm0.16^{a}{}_{1}$	58 ±5.5	55.4±5.1	-2.6±0.6
DW10	25±0.30	23±0.87	-1.93±0.74	36.5±0.3	34.9±0.1	-1.56±0.32	63.4±2.8	58.4±2.9	-5±0.1.14
CQ25	23.6±0.3	25±0.75	$1.65\pm0.68^{a}{}_{1}$	36.6±0.1	36.4 ±0.2	$-0.18\pm0.25^{a}{}_{3}$	64.8±3.4	63.8±3.6	$-1.0\pm0.45^{a}{}_{2}$

Results are expressed as mean ±SEM, n=5; a = compared to negative control (DW=Distilled Water); 1= $p < 0.05$; 2 = $p < 0.01$; 3= $p < 0.001$; CS = *Clematis simensis.* B.Wt = Body Weight; CQ=Chloroquine

Effect of the fractions on parasitemia and survival time in the 4-days suppressive test

Despite a dose dependent increase in antiplasmodial activity, the aqueous as well as n-hexane fractions of *C. simensis* didn't have significant activity when compared to the vehicle treatment. The chloroform fraction at 400mg/kg was able to suppress parasitemia by 26.5% which was significant when compared to vehicle ($p<0.01$) and the 100 mg/kg dose ($p<0.05$) treatments. The standard drug chloroquine was able to completely clear the parasitemia. In the absence of significant antiplasmodial activity, treatment with the aqueous fraction of *C. simensis* at 400 mg/kg dose and n-hexane fraction at 400mg/kg doses have a significant increase in survival of mice when compared to the vehicle with respective $p<0.01$ and $p<0.05$ values, Table 4.

Table4. Effect of the fractions on parasitemia and survival time in the 4-days suppressive test

	Treatment(mg/kg)	% Parasitemia	% suppression	Survival time (day)
	CS 100	62.2±4.81	9.1	5.8±0.58
	CS 200	53.5±4.17	21.8	7.6±0.51
	CS 400	53.2±5.15	22.2	$8.8\pm0.86^{a}{}_{2}{}^{b}{}_{2}$
	DW 10	68.4±2.71	0	5.4±0.24
	CQ 25	$0.00\pm0.00^{\infty}{}_{3}$	100	$30\pm0.00^{\infty}{}_{3}$
n-Hexane fraction	CS 200	42.7±2.9	3	7.4±0.24
	CS 400	40.3±2.3	8.4	$7.6\pm0.24^{a}{}_{1}$
	Tween-80	44±6.4	0	6.6±0.24
	CQ 25	$0.00\pm0.00^{\infty}{}_{3}$	100	$30\pm0.00^{\infty}{}_{3}$
Chloroform Fraction	CS 100	71.0±4.04	2.2	6.2±0.49
	CS 200	59±5.41	18.7	6.8±0.58
	CS 400	$53.4\pm3.6^{a}{}_{2}{}^{b}{}_{1}$	26.5	$7.2\pm0.86^{a}{}_{3}{}^{b}{}_{1}$
	Tween-80	72.6±1.36	0	5.0±0.32
	CQ 25	$0.00\pm0.00^{\infty}{}_{3}$	100	$30\pm0.00^{\infty}{}_{3}$

Results are expressed as mean ±SEM, n=5; a = compared to negative control (DW-distilled water and 7% Tween-80suspension; b= compared to100mg/kg; 1= $p < 0.05$; 2 = $p < 0.01$; 3= $p < 0.001$; ∞ = all treatments; CS = *Clematis simensis;* CQ=Chloroquine

Effect of fractions on body weight, rectal temperature and PCV measurements in 4-days suppressive test

As shown in Table 5, there was a dose dependent decrease in body weight loss for all fractions of *C. simensis* treatment compared to the negative controls. Yet, these were not statistically significant different. The same effect was also noted regarding rectal temperature for the aqueous and n-hexane fractions. In contrast, the chloroform fraction was able to demonstrate a significant prevention of decrease in rectal temperature ($p<0.05$) for both the 200mg/g as well as the 400mg/kg dose treatments.

Though PCV decrease was largely prevented in a dose dependent manner with treatments of all fractions of *C. simensis*, all were not significantly different compared to the vehicle treatment. In the three parameters (body weight, rectal temperature and packed cell volume changes), chloroquine treatment was able to prevent the decrease at significant level specially compared to the vehicle treatments.

Table5. Effect of fractions on body weight, rectal temperature and PCV measurements in 4-days suppressive test

Extract	Treatment (mg/kg)	Body weight(g)			Rectal temperature(°C)			Packed cell volume (PCV)		
		B.Wt at D0	B.Wt at D4	% change	T at D0	T at D4	% change	PCV at D0	PCV at D4	% change
Aqueous fraction	CS100	23.8±0.5	21.9±0.6	-1.85±0.95	36.9±0.1	34.4±0.4	-2.56±0.51	59.2±3.8	52.6±5.3	-6.6±1.8
	CS200	26.4±0.4	25.1±0.8	-1.35±0.63	36.9±0.1	34.9±0.4	-2.00±0.48	60.4±4.3	54±5.5	-3.6±2.3
	CS400	27.3±0.1	26.1±0.5	-1.27±0.4	37±0.10	35±0.20	-1.98±0.11	59.2±6.4	54.2±8.1	-5±2.51
	DW10	27.8±0.2	25.5±0.5	-2.32±0.55	37.3±0.2	34.6±0.3	-2.7±0.38	61.2±1.7	54.2±1.9	-7.0±0.45
	CQ25	27.6±0.2	27.9±0.2	$0.3\pm0.095^{a}{}_{1}$	36.9±0.3	36.8±0.3	$-0.10\pm0.18^{a}{}_{2}{}^{b}{}_{2}{}^{c}{}_{1}{}^{d}{}_{1}$	56.8±3.2	57.4±3.2	$0.6\pm0.4^{a}{}_{1}$
n-Hexane fraction	CS200	25.1±0.5	23.5±0.6	-1.6±0.3	37.5±0.2	35±0.2	-2.5±0.2	44.2±3.4	41±3.5	-3.2±0.4
	CS400	26.1±0.4	24.8±0.6	-1.3±0.2	36.9±0.2	34.9±0.1	-2.00±0.2	47.4±2.0	44.6±2.3	-2.8±0.8
	Tween-80	26.8±0.4	24.7±0.2	-2.14±0.4	36.5±0.4	33.9±0.1	-2.5±0.4	52±1.0	46.8±1.9	-5.2±1.1
	CQ25	25.1±0.7	25.5±0.7	$0.4\pm0.09^{a}{}_{3}{}^{b}{}_{3}{}^{c}{}_{2}$	36.5±0.3	36.9±0.4	$0.4\pm0.2\infty_{3}$	53.4±2.7	52.6±2.4	$-0.8\pm0.37^{a}{}_{2}$
Chloroform fraction	CS100	27.5±0.2	24±0.3	-3.43±0.26	37.3±0.3	34.8±0.3	-2.5±0.5	70.6±2.2	63±3.3	-7.6±1.3
	CS200	26.6±0.8	24.7±1.4	-1.9±2.15	36.7±0.2	35.5±0.4	$-1.28\pm0.5^{a}{}_{1}$	70.0±1.4	63.2±3	-6.8±1.9
	CS400	27.6±0.3	26.4±1.2	-1.22±1.33	36.9±0.1	35.7±0.1	$-1.2\pm0.18^{a}{}_{1}$	73.2±2.1	67.4±2.9	-5.8±1.16
	Tween-80	27.3±0.4	23.3±0.8	-4.0±0.5	37.7±0.3	34.8±0.1	-2.94±0.35	55±0.3	46±2	-9.0±1.8
	CQ25	27.1±0.5	28.2±0.4	$1.1\pm0.09^{a}{}_{1}$	37.2±0.3	37±0.3	$-0.14\pm0.098^{a}{}_{3}{}^{b}{}_{2}$	52±0.9	52.6±0.9	$0.6\pm0.24^{a}{}_{2}$

Results are expressed as mean ±SEM, n=5; a = compared to negative control (DW-distilled water and 7% Tween-80suspension; b=compared to100mg/kg; c=compared to 200mg/kg; d=compared to400mg/kg; 1= $p < 0.05$; 2 = $p < 0.01$;3= $p < 0.001$; ∞ = all treatments; CS = *Clematis simensis;* CQ=Chloroquine; B.Wt =Body Weight; PCV=Packed Cell Volume

Effect of chloroform fraction on parasitemia and survival time in the Rane's test

The effect of chloroform fraction of *C. simensis* on parasitemia and survival of the mice following Rane's model is shown in Table 6. The parasitemia level was significantly inhibited ($p<0.001$) by the 600mg/kg dose treatment than the vehicle and lower dose extract treatments. The same is true regarding the increase in survival time.

Table6. Effect of chloroform fraction on parasitemia and survival time in the Rane's test

Extract	Treatment (mg/kg)	%Parasitemia at D6	%Parasitemia at D7	%Parasitemia at D8	% inhibition	Survival time (day)
Chloroform Fraction	CS 200	19.8±0.97	22 ±1.05	23.0±1.05	1.71	6.6±0.24
	CS 400	17.6±1.89	$18.4\pm1.4^{a}{}_{1}$	$19.0\pm1.18^{a}{}_{1}{}^{b}{}_{1}$	18.8	$8.4\pm0.4^{a}{}_{3}{}^{b}{}_{2}$
	CS 600	$13.8\pm0.73^{a}{}_{2}{}^{b}{}_{1}$	$10.2\pm0.66^{a}{}_{3}{}^{b}{}_{3}{}^{c}{}_{3}$	$9.4\pm0.68^{a}{}_{3}{}^{b}{}_{3}{}^{c}{}_{3}$	59.8	$10.8\pm0.37^{a}{}_{3}{}^{b}{}_{3}{}^{c}{}_{3}$
	Tween-80	21±1.14	22.8±0.97	23.4±0.75	0	5.6±0.24
	CQ 25	$3\pm0.16^{\infty}{}_{3}$	$1.02\pm0.27^{\infty}3$	$0.00\pm0.00^{\infty}{}_{3}$	100	$30\pm0.000^{\infty}{}_{3}$

Results are expressed as mean ±SEM, n=5; a = compared to negative control (7% Tween-80suspension); b=compared to100mg/kg; c=compared to 200mg/kg; 1= $p < 0.05$; 2 = $p < 0.01$; 3= $p < 0.001$; ∞ = all treatments; CS = *Clematis simensis;* CQ=Chloroquine

Effect of chloroform fraction on body weight, rectal temperature and PCV measurements in Rane's test

The 400mg/kg and 600mg/kg doses of the chloroform fraction were able to prevent body weight losses with respective $p<0.01$ and $p<0.001$ values compared to vehicle treatment. Table7. The same significant prevention was observed for prevention of rectal temperature decrease for the two doses. Both doses were again able to prevent a significant decrease in PCV ($p<0.01$) compared to the vehicle treatment.

Table7. Effect of chloroform fraction on body weight, rectal temperature and PCV measurements in Rane's test

Extract	Treatment (mg/kg)	Body Weight			Rectal Temperature			Packed Cell Volume		
Chloroform fraction		B.Wt at D0	B.Wt at D4	% change	T at D0	T at D4	% change	PCV at D0	PCV at D4	% change
	CS200	27.5±0.2	24±0.3	-3.39±0.44	37.3±0.3	34.8±0.3	-7.4±0.53	70.6±2.2	58.6±6.5	-10.3±2.2
	CS400	26.6±0.8	24.7±1.4	$-2.58\pm0.17^{a}{}_{2}$	36.7±0.2	35.5±0.4	-6.7 ±0.32a2	70±1.4	61.6±6.2	-7.3±0.54a2
	CS600	27.6±0.3	26.4±1.2	$-1.9\pm0.25^{a}{}_{3}{}^{b}{}_{2}$	36.9±0.1	35.7±0.1	-4.98±0.32a3b2	73.2±2.1	67.4±2.9	-5.1±0.32a2
	Tween-80	27.3±0.4	24.1±0.1	-4.1±0.23	37.7±0.3	34.8±0.1	-8.13±0.49	55±0.3	50.4±0.7	-15.5±2.5
	CQ25	27.1±0.5	27.4±0.5	$0.5\pm0.07^{\infty}{}_{3}$	37.2±0.3	37.1±0.3	0.06±0.07∞3	52±0.9	52.6±0.9	-1.52±0.22a3b2

Results are expressed as mean ±SEM, n=5; a = compared to negative control (7% Tween-80suspension); b= compared to100mg/kg; 2 = $p < 0.01$; 3= $p < 0.001$; ∞ = all treatments, CS = *Clematis simensis;* CQ=Chloroquine ; B.Wt =Body Weight; PCV=Packed Cell Volume**.**

IV. Discussion

The findings of the present study indicate that the extract of the root of *C. simensis* possesses anti-malarial activity. To reveal this outcome, the methods and procedure followed were built on strong justifications. Several solvents with differing polarity were used for extraction since this allows obtaining diverse phytochemicals [43]. The *in vivo* model offers possibility of having pro-drug effect as well as involvement of the immune system in enhancing anti-malarial activity of the plant [44]. Mouse model is mostly used for assessment of antiplasmodial activities of plant extracts since there is reasonably high homology and similarity between mammalian genetics and physiology [43]. Regarding *Plasmodium berghei*, there is a rich experience in using it for screening anti-malarial activity of potential plants [36, 45, 46] and essential anti-malarial agents such as chloroquine and artemisinins came through the use of a similar model [47]. In 4 day suppressive test and Rane's test methods, determination of percent inhibition of parasitemia is the most reliable parameter. A mean parasitemia level ≤ 90% to that of mock-treated control animals usually indicates that the test compound is active in standard screening studies [48]. Chloroquine, though no longer a first-line drug in the treatment of malaria, is used as control drug in this study because the *Plasmodium* parasite used for inoculation is a chloroquine-sensitive strain [49]. It clears parasitemia to undetectable levels, 0% in both crude and solvent fractions of *C. simensis* as with other studies [36, 45].

The highest yield observed with the aqueous fraction may be due to high concentration of polar compounds in the root of the plant species that better dissolve in water. A previous study on the leaf part demonstrated a better yield using 80% methanolic solvent [28]. In the era of increased extinction risk of medicinal plants due to several reasons such as routine collection of root parts, the smaller yield observed in the present study when compared to the leaf, may discourage the practice.

No death was caused by an oral dose of 2000 mg/kg body weight of the hydro-methanolic extract of the leaves of *C. simensis* could imply the safety of the plant to be used in the treatment of malaria. It reiterates findings of Tadele et al on the same plant [28]. This is consistent with several previous studies screening anti-malarial and other activities of medicinal plants in Ethiopia [46, 50-53].

With the 4 days suppressive test, the crude extract demonstrated a dose dependent antiplasmodial activity which was significant at the 600mg/kg dose when compared to the vehicle. This is consistent with previous studies screening anti-malarial activities [50, 54]. Maximal effect observed at this dose might be due to higher level and composition of active compounds [42]. Despite a dose dependent increase in antiplasmodial activity, the aqueous as well as n-hexane fractions of *C. simensis* did not have significant activity when compared to the vehicle treatment. It probably was due to less presence of active phytochemicals in these fractions. Though insignificant, the greater than 10% parasite inhibition by the 200mg/kg and 400mg/kg doses of the aqueous fraction compared to the vehicle shows that the plant is active in the present evaluation [48]. The chloroform fraction was also shown to possess a significant activity at the 400mg/kg dose ($p<0.01$). The active ingredients of this plant responsible for its anti-malarial activities may be localized in this fraction as with previous studies [36]. Regarding parasitemia level, the standard drug maintained complete clearance unlike all extract treatments. This is consistent with previous studies [36, 45].

Survival of mice was increased in dose dependent manner with all treatments of the extract and was significant ($p<0.001$) for both the 600mg/kg of the crude extract and the 400mg/kg of the chloroform fraction. These effects may be attributed to the parallel increase in parasite suppression effect.

Anemia, rectal temperature reduction, and reduction in body weight are the general features of malarial infection in mice and human malarial infections [55, 56]. This means that a potent anti-malarial is expected to improve anemia, prevent body weight loss, and stabilize temperature in infected mice with increasing parasitemia. PCV was measured to determine the effectiveness of *C. simensis* root in preventing malaria-induced hemolysis. The crude extract and fractions at all doses did not prevent a decrease in PCV. Though insignificant compared to the vehicle, the highest doses of the extract reduced the magnitude of PCV fall to the highest level. This may be correlated with the observed effect on parasitemia level that is decreasing but not completely cleared. Thus parasite continues to reduce PCV level. This decrease might largely be due to the parasitemia level. This is because the saponins, widely known to cause hemolysis, are absent in our plant [56, 57].

Regarding the decrease in body weight of the mice, only the standard drug showed a reversal of this effect. Whereas extract treatment was demonstrated to reduce the magnitude of decrease in weight though statistically insignificant. The highest effect was observed at the largest doses. This may attributed to inability of the extract to maintain complete clearance of parasitemia level. The standard drug continued to have a significant increase in body weight compared to the vehicle. The most effective doses (600mg/kg of the crude extract and 400mg/kg of the chloroform fraction) showed a significant prevention of a decrease in temperature compared to vehicle treatment.

The findings from the curative test (established infection) imply that the extract was able to reduce the parasitemia level only at the 600mg/kg dose of the chloroform fraction. This was significant ($p<0.001$)

compared to vehicle and lower dose extract treatments. This suggests that the active phytochemicals might be concentrated in chloroform fraction [36].

Taken all together, the above findings suggest that the study plant is active against malaria in the 4 days suppressive as well curative tests according to the standard that greater than 10% pasitemia suppression compared to vehicle treatment is indicative of this outcome [48]. These effects may be additive and bring successful therapy of malaria using the plant in the Ethiopian folk medicine. It may be possible to consider the plant as a potential source of anti malarial agents, as it is desirable to have both activities in a potential phytodrug [58].

Our study showed that *C. simensis* root has the following secondary metabolites which have been suggested to be responsible for antiplasmodial activity of other plants; alkaloids, flavonoids, terpenoids and phenols. Alkaloids such as quinine [57], sesquiterpene trioxane lactone (Artemisinin) [59], flavonoids [60], and phenols [61, 62] are known to have anti-malarial activities. Therefore, the observed effect could be associated with the above four phytochemicals in combination.

Although the mechanism of action of these extracts has not been elucidated, some plants are known to exert antiplasmodial activity via antioxidant activity. Plants having a high concentration of phenolic compounds like phenol itself and flavonoids (poly-phenols) are a reservoir of novel antioxidant molecules [63, 64]. A previous study showed the hepatoprotective (antioxidant) effect of *C. chinensis* extract, member of the genus clematis [65].

As earlier reported, many anti-malarial herbal remedies may exert their anti-infective effects not only by directly affecting the pathogen, but also by indirectly stimulating natural and adaptive defence mechanisms of the host by other mechanisms. Therefore, extracts that can stimulate innate and/or adaptive immunity may be able to contribute to prophylaxis and treatment not only for malaria but for other diseases as well [66, 67].

The anti-inflammatory activity reported so far for the plant [28] may also contribute for the observed anti-malarial effect of the plant. Therefore, further studies on *C. simensis* shall be conducted to identify novel agents as new anti-malarial drugs and their mechanism of actions shall be explored.

V. Conclusion

The results of this study showed absence of acute toxicity at 2000mg/kg dose during ingestion and that hydro alcoholic crude extract and chloroform fraction possess anti-malarial activities. This effect may be attributed to alkaloids, phenols, terpinoids and flavunoid detected in the plant. Therefore the folkloric anti-malarial claim of the plant is validated. This plant could be further studied in different models and isolation of phytochemicals may lead the development of new plant-based anti-malarial agent.

List of abbreviations

EHNRI: Ethiopian Health and Nutrition Research Institute
MST: Mean Survival Time
PCV: Packed Cell Volume
WHO: World Health Organization

Declarations

Ethics approval and consent to participate: Ethical clearance was secured from the Institutional Review Board of the University of Gondar.

Consent for publication: Not applicable.

Availability of data and material: All data generated or analyzed during this study are included in this article.

Competing interests: The authors declare that they have no competing interests.

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Authors' contributions: All authors involved in the design of the study. Maru Asmare (MA1) conducted the actual study and the statistical analysis. DAG2 and MA2 supervised the experiment. While MA1 drafted the manuscript, DAG2 and MA2 edited it. All the authors read and approved the submitted version of the manuscript.

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