

# Evaluation of Antimalarial Activity of Hydromethanolic Crude Extract and Solvent Fractions of the Leaves of *Nuxia congesta* R. Br. Ex Fresen (*Buddlejaceae*) in *Plasmodium berghei* Infected Mice

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**Background:** In Ethiopia, malaria control has been complicated due to resistance of the parasite and its vectors to the current drugs. Therefore, new drugs are required to avert the problem posed by drug-resistant *Plasmodium* strains. There is need to investigate alternative sources of antimalarial agents and plants are potential source of antimalarial drugs. This study aimed to investigate the antimalarial activity of the leaves of *N. congesta* crude extract (hydromethanolic extract) and solvent fractions (n-hexane, chloroform, and aqueous fractions of crude extract) traditionally used to treat malaria in many parts of Ethiopia.

**Methods:** Acute oral toxicity of the leaves of *N. congesta* extract was assessed in mice up to a dose of 5,000 mg/kg body weight. Antiplasmodial activities of crude extract and solvent fractions were assessed in *P. berghei* infected female Swiss albino mice models using the Peter's 4-day suppressive test. The curative activities of crude extract and fractions were evaluated using Rane's test.

**Results:** Plant extract exhibited no signs of toxicity on mice at a dose of 5,000 mg/kg body weight. Crude extract showed significant parasitemia suppressions at doses of 500 mg/kg ( $P<0.05$ ), 750 mg/kg ( $P<0.01$ ), and 1,000 mg/kg ( $P<0.001$ ) as compared to negative control in the Peter's 4-day suppressive test, but failed to reach a significant level at 500 mg/kg and 750 mg/kg in the curative test (Rane's test). Aqueous fraction showed significant parasitemia suppression at a dose of 400 mg/kg ( $P<0.05$ ) in curative test and 600 mg/kg ( $P<0.05$ ) in Peter's 4-day suppressive and curative tests. Maximum suppressive effects of extract (58.13%) and aqueous fraction (44.9%) were observed at the highest doses administered.

**Conclusion:** Hydromethanolic leaf extract of *N. congesta* and its aqueous fraction exhibited antimalarial activities. The antimalarial activity and lack of acute toxicity are suggested to uphold the earlier claims made by the Ethiopian traditional practitioners.

**Keywords:** parasitemia, antimalaria, extract, suppression, curative

## Introduction

Malaria is the main widespread disease in tropical and sub-tropical regions of the world affecting humans.<sup>1,2</sup> It exerts a great economic burden on endemic countries.<sup>3</sup> Africa is often called the "heartland" of malaria and remains a major public health threat and obstacle to economic development.<sup>4</sup> According to a World Health

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Organization (WHO) report in 2018, 219 million cases of malaria occurred worldwide in 2017.<sup>5</sup> In Ethiopia, malaria affects more than 68% of the population, from which *P. falciparum* accounted for nearly 55% of all malaria cases in 2013.<sup>6</sup> According to a report in 2017, malaria caused an estimated 30,323 deaths and 621,345 new cases in 2015. It accounts for 6% of malaria cases and 12% of the cases and deaths due to *Plasmodium vivax* globally.<sup>7</sup>

Development of resistant strains of *Plasmodium*, particularly *P. falciparum* to currently available drugs, has posed a great challenge in the treatment and prevention of malaria.<sup>8</sup>

About 70% of the African population rely on traditional medicine, since affordable conventional medicines are inaccessible.<sup>9</sup> In rural areas of Ethiopia people resort to use traditional herbal medicine to treat malaria since rural health centers are few and poorly staffed. Traditionally, *N. congesta* is one of the medicinal plants used for treatment of malaria in Sidama district, Ethiopia.<sup>10</sup>

Medicinal plants are a potential source of new antimalarial drugs since they contain a lot of metabolites with a great variety of structures and pharmacological activities.<sup>11</sup> From previous reports, most medicinal plants which have secondary metabolites in their crude extracts and solvent fractions showed antiplasmodial activities.<sup>12</sup> N-butanol fraction of *Dodonaea angustifolia*,<sup>13</sup> aqueous leaf extracts of *Markhamia tomentosa* and *Polyalthia longifolia*,<sup>12</sup> chloroform extract of *Croton macrostachyus*,<sup>14</sup> and *Osiris quadripartite*<sup>15</sup> are illustrative examples. Among 40 species of *Nuxia*<sup>16</sup> some species are used to treat malaria in Madagascar.<sup>17</sup> *Nuxia oppositifolia* is used to treat malaria, headache, and enlarged spleen and cough,<sup>18</sup> while *N. sphaerocephala* is used to treat malaria associated splenomegaly in Madagascar.<sup>16</sup>

*N. congesta* R. Br. ex Fresen (*Buddlejaceae*), “Atquaro” (Amharic), “Nole” (sidama), and “Common wild elder/brittle wood” (English) is a member of the winter-flowering evergreen small tree or shrub.<sup>19</sup> Ethnobotanical study showed that *N. congesta* has a wide range of uses. In Ethiopia, the whole leaf part of the plant is used for local beer making.<sup>20</sup> Fresh leaves, stem bark, and flower part are used for treatment of tonsillitis, dermatological problems, management of rheumatism, and joint swelling<sup>(21,23–)</sup>. In Kenya, the boiled roots of *N. congesta* are used for the treatment of common colds, flu, and abdominal pain.<sup>20</sup> Bark is used for treatment of indigestion, menstrual problems, and as an expectorant. Bark and leaves are also chewed against indigestion, whereas the ash of leafy twigs in water is used to treat diarrhea. In

Uganda, the bark is used to treat toothache and leaf smoke is used to treat mastitis in cattle.<sup>18</sup>

In many parts of Ethiopia, particularly in Sidama district, the leaf of the plant is used to treat malaria symptoms, with no scientific evidence of its safety or efficacy. This study was designed to assess acute oral toxicity and antimalarial activity of the leaves of *N. congesta* hydro-methanolic extract and its solvent fractions in *P. berghei* infected mice.

## Materials and Methods

### Materials

#### Plant Material

Fresh leaves of *N. congesta* were harvested from their natural habitat in “*Chilga woreda*,” North Gondar zone, Northwest Ethiopia in January, 2017. Leaves were wrapped with plastic sheets during transport. Plant identification and voucher specimen referencing were done at the Department of Biology herbarium in the College of Natural and Computational Sciences, University of Gondar by a botanist (Mr. Abyu Enyew). The plant specimen was deposited in the University’s Herbarium with a voucher number MF 001.

#### Experimental Animals and Parasite

Female Swiss albino mice (23–32g), obtained from the Department of Pharmacology Animal House, University of Gondar were used for the acute oral toxicity and antiplasmodial activity testing. All experimental animals were housed under standard environmental conditions of temperature at 22–24°C under a 12 hour dark–light cycle and allowed free access to drinking water and standard pellet diet. Animal attendants maintained hygiene by constant cleaning and removal of feces from cages every 4 days. Before the actual experiment, animals were acclimatized for 1 week to the experimental environment. The care and handling of the experimental animals was according to the national research council guide for the care and use of laboratory animals.<sup>24</sup> Ethical approval for the study was obtained from the Department of Pharmacology/University of Gondar Ethics and Research Committee, Gondar, Ethiopia, with reference number SOP4/78/09.

### Method

#### Extraction

Fresh leaves of *N. congesta* were washed using tap water, dried under shade, and pulverized to coarse powder using a mortar and pestle. The powder (1.224 kg) was exhaustively

macerated in 80% methanol (7.2 L) for 72 hours with occasional shaking. Then, it was filtered using a tea sieve and Whatman filter paper No.1 (Whatman, England). The filtrate was kept in a refrigerator at +4°C. The residue was re-macerated for 72 hours and this procedure was repeated for the third time.<sup>14,25,26</sup> The filtrates were combined and concentrated using a rotary evaporator (RE200, Germany) at 45 rpm and 40°C; then, it was dried using a freeze drier (Ningbo-Scientz-Biotechnology) and the dry product was kept in a desiccator until use.<sup>14,27</sup>

### Fractionation

Antimalarial activity evaluation of the crude extract revealed *N. congesta* methanol extract to have a better activity (section 3.3) and further fractionation was pursued using this plant. The dried extract (160 g) was suspended in distilled water (800 mL) and then successively partitioned in n-hexane and chloroform using a separatory funnel. The n-hexane and chloroform fractions were concentrated under reduced pressure using a rotary evaporator (RE200, Germany) and dried in an oven at 40°C while the aqueous fraction was dried using a freeze drier (Ningbo-Scientz-Biotechnology). The dried fractions were placed in a refrigerator at +4°C until use to prevent decomposition.<sup>10,23,25</sup>

### Phytochemical Screening

Eighty percent methanol leaf extract of *N. congesta* was screened for the presence of secondary metabolites using the method described by Azwanida 2015; Fentahun and Makonnen 2017; Fidock 2004.<sup>25,28,29</sup>

### Acute Oral Toxicity

The acute oral toxicity of *N. congesta* leaf extract was evaluated according to the procedures outlined by the Organization for Economic Co-operation and Development (OECD) 425.<sup>30</sup> The hydromethanolic extract was suspended in a vehicle (distilled water). Following a 3 hour fasting period, eight mice were divided into groups of four (one, one, two, and four mice per group). Extract doses were calculated in reference to the body weight of mice and each mouse was treated with a single oral dose of the extract followed by withholding food for 1 hour. The first group was treated with 2,000 mg/kg of extract, while the second group was treated with 5,000 mg/kg followed by observation for any signs of toxicity. Since death was not observed in the first and second groups within 24 hours after administration of the extract, the third and fourth groups were treated with 2,000 mg/kg and 5,000 mg/kg *N. congesta* leaf extract. Mice were observed continuously for 1 hour after administration of

the extract; intermittently for 4 hours, over a period of 24 hours and then frequently for 24 hours for 14 days. Gross behavioral changes such as loss of appetite, hair erection, lacrimation, tremors, convulsions, salivation, diarrhea, mortality, and other signs of toxicity manifestation were observed and recorded.

### Parasite Infection of Experimental Animals

The chloroquine sensitive strain of *P. berghei* (strain ANKA) was generously donated by the Ethiopian Public Health Institute, Addis Ababa as cryo-frozen stock of parasitized red blood cells (PRBCs) from female Swiss albino mouse (*Mus musculus*). The parasite was maintained by serial passage of blood from infected to non-infected mice within 5–6 days of infection.<sup>23,31</sup> Donors with a parasitemia level of 20–30% were sacrificed after anesthetizing with ether and blood collected by cardiac puncture into heparinized tubes. The blood was then diluted with phosphate buffered saline (PBS) based on the parasitemia level of each donor and the RBC count of normal mice such that 1 mL blood contained  $5 \times 10^7$  parasites. The experimental animals were each treated with  $1 \times 10^7$  PRBCs by IP injection. The parasite was maintained by transferring parasitized erythrocytes from infected mice to non-infected mice every 5–6 days for the subsequent experiments.<sup>23</sup>

### Grouping and Dosing of Animals

#### Suppressive Test

In order to evaluate the chemosuppressive effect of crude extract, infected mice were randomly assigned to eight groups, each consisting of five mice. Groups I, II, III, IV, V, and VI were orally treated with 100, 200, 400, 500, 750, and 1,000 mg/kg of *N. congesta* hydroalcoholic leaf extract, respectively, while groups VII and VIII were treated with negative (water; 10 mL/kg) and positive controls (chloroquine; 25 mg/kg), respectively. Similarly, 40 mice were randomly divided into eight groups of five each to evaluate chemosuppressive chloroform and n-hexane fractions. Groups I, II, and III were orally treated with 100, 200, and 400 mg/kg of chloroform and groups IV, V, and VI were treated with n-hexane fraction. Negative control (3% T80; 10 mL/kg) and positive control (chloroquine; 25 mg/kg) were administered to groups VII and VIII, respectively. Additionally, to evaluate the aqueous fraction, six groups of infected mice each consisting of five mice were assigned. Groups I, II, III, and IV were treated with 100, 200, 400, and 600 mg/kg while groups V and VI were

given negative control (water; 10 mL/kg) and positive control (chloroquine; 25 mg/kg), respectively.

### Curative Test

Forty mice were randomly divided into eight groups of five each to evaluate the curative effect of the leaves of *N. congesta* hydromethanolic extract its aqueous fraction. Groups I, II, and III were treated with 500, 750, and 1,000 mg/kg of hydromethanolic extract and groups IV, V, and VI were treated with 200, 400, and 600 mg/kg of aqueous fraction. Groups IV and V were treated with negative control (water; 10 mL/kg) and positive control (chloroquine; 25 mg/kg), respectively.

### Chemosuppressive Test (Peter's 4-Day Suppressive Test)

To evaluate the chemosuppressive activities of crude extract and solvent fractions in *P. berghei* (ANKA) infected mice the method described by Fidock et al<sup>31</sup> and Tarkang et al<sup>32</sup> was used.

On the first day (D0), the mice were each infected with  $1 \times 10^7$  *P. berghei*. Three hours later, the experimental groups of mice were each treated orally with 10 mL/kg of control or extract. Treatment was carried out for 4 consecutive days (D0–D3). The body weight and temperature of each mouse were measured on the first day (D0) and on the fifth day (D4).

On the fifth day (D4), thin blood film was prepared from the tail blood of each experimental animal, fixed in methanol, and stained with Giemsa to reveal parasitized erythrocytes. Parasitemia was determined by using a light microscope with a 100X objective lens, and percent parasitemia was calculated using the following equation:<sup>23</sup>

$$\% \text{Parasitemia} = \frac{\text{No. of parasitized RBC}}{\text{Total No. of RBC counted}} \times 100$$

Average percentage chemosuppression was calculated as

$$\text{Percentage chemosuppression} = 100 - \frac{\text{Mean parasitemia (crude extract/fraction)treated}}{\text{Mean parasitemia(water/T80)control}} \times 100$$

The mean survival time (MST) of the mice in each treatment group was determined over a period of 29 days (D0–D28) as follows:<sup>23</sup>

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

### Curative Test

Evaluation of the curative potential of the crude extract and aqueous fraction was carried out in established

infection.<sup>14,32</sup> Forty mice were infected with *P. berghei* ( $1 \times 10^7$ ) by IP injection on the first day (D0). Seventy-two hours later (D3), the mice were randomly divided into eight groups of five mice each.

Extract, aqueous fraction, and controls were administered once daily for 5 days. Giemsa-stained thin smears were prepared from tail blood samples collected on each day of treatment to monitor the parasitemia level. The body weight and temperature were taken before infection (D0) and from the fourth day (D3) to the eighth day (D7), while the MST of the mice in each treatment group was determined over a period of 29 days (D0–D28) using a formula described in section 2.2.7 above.<sup>32,33</sup>

### Determination of Packed Cell Volume in Suppressive and Curative Tests

Packed cell volume was determined before infection (D0) and after infection (D4) to predict the effectiveness of crude extract and solvent fractions in preventing hemolysis resulting from increasing parasitemia associated with malaria. Heparinized capillary tubes were used for collection of blood from the tail of each mouse. Blood was collected up to 75% of the volume of the capillary tube (capacity of the capillary tube is 0.1 mL; 0.075 mL blood) and sealed with clay sealant. The tubes were placed in a micro-hematocrit centrifuge with the sealed end outwards and centrifuged for 10 minutes at 11,000 rpm. The tubes were taken out of the centrifuge, the volume of erythrocytes was measured using a standard Micro-Hematocrit Reader, and PCV was determined as follows.<sup>14,34</sup>

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

### Statistical Analysis

Data of the study were presented as mean±SEM. Comparisons of parasitemia suppression, body weight, body temperature, and survival time of animals were analyzed using SPSS Windows version 20.0. Statistical significance testing was done by using one-way analysis of variance (ANOVA) followed by Post-hoc Tukey's test. *P*-values of less than 0.05 were considered statistically significant.

### Ethical Clearance

Animals were handled according to the guidelines for care and use of laboratory animals and OECD-guidelines.<sup>32</sup> Ethical approval for the study was obtained from the Department of Pharmacology/University of Gondar Ethics and Research Committee, Gondar-Ethiopia (SOP4/78/09).

## Results

### Phytochemical Characterization

Extraction and solvent fractionation of the leaves of *N. congesta* produced 355 g (29%), 18.56 g (11%), 8 g (5%), and 133.6 g (83.5%) of crude extract, chloroform, n-hexane, and aqueous fractions, respectively. Aqueous, chloroform, n-hexane fractions, and crude extract were brown, green, dark green, and dark brown in color, respectively. As indicated in Table 1, phytochemical analysis revealed the presence of saponins, steroids, phenol, flavonoids, cardiac glycosides, terpenoids, and tannins, while alkaloids and anthraquinones are absent.

### Acute Oral Toxicity Test

The leaves of *N. congesta* hydromethanolic extract did not cause any signs of adverse effect and/or mortality at 2,000 and 5,000 mg/kg oral doses throughout the 14-day study period. Adverse effects (visible behavioral changes) such as hair erection, lacrimation, urination, salivation, weakness, and reduction in their locomotion and feeding activities were not observed within the first 24 hours of extract administration. No significant changes were observed in their body weights. The estimated maximum tolerable dose was above 5,000 mg/kg body weight for the crude extract tested.

### Evaluation of Suppressive Activity of *N. Congesta* Leaf Extract

*N. congesta* Leaf Extract showed dose dependent chemosuppressive activity on parasitemia (Table 2, Figures 1 and 2). The effects were statistically significant ( $P < 0.01$ ) at doses of 500 mg/kg, 750 mg/kg, and 1,000 mg/kg as compared to the negative control. This result showed that the survival times of crude extract treated mice were increased in a dose dependent

manner although statistical significance was reached at higher doses of 500 mg/kg ( $P < 0.05$ ), 750 mg/kg, and 1,000 mg/kg ( $P < 0.01$ ).

The protection of body weight loss by the crude extract was comparable with the positive control (Chloroquine) at the highest dose (1,000 mg/kg). Crude extract protected body temperature reduction of mice dose dependently but was not statistically significant as compared to the negative control. Only the positive control showed significant protection in temperature reduction ( $P < 0.05$ ) as compared to the negative control.

### Evaluation of Suppressive Activity of N-Hexane, Chloroform, and Aqueous Fractions of *N. Congesta* Leaves

The aqueous fraction showed a dose dependant parasitemia suppression effect, 16.27%, 20.57%, 23.34%, and 44.88% at a dose of 100 mg/kg, 200 mg/kg, 400 mg/kg, and 600 mg/kg, respectively, while n-hexane and chloroform fractions fail to have this effect (Table 3, Figures 3 and 4). However, significant parasitemia suppression was reached at 600 mg/kg ( $P < 0.05$ ) as compared to the negative control. All doses of aqueous fraction increased the survival days of mice significantly ( $P < 0.05$ ) as compared to the distilled water treated group. Chloroform fraction showed 15% parasitemia suppression at 200 mg/kg and the survival time reached a significant level at this dose as compared to the negative control (T80). The parasitemia suppression effect and prolonged survival were not observed in n-hexane fraction treated groups.

Aqueous fraction exhibited a dose-dependent protection in body weight reduction. In the n-hexane fraction treated groups, there was loss in body weight and body temperature elevation. Chloroform fraction showed dose dependent body weight reduction of experimental animals.

### Effect of *N. Congesta* Leaf Extract and Aqueous Fraction on Packed Cell Volume in Suppressive Test

The effects of the crude extract and aqueous fraction on packed cell volume at day 0 and 4 are presented in Table 4. Crude extract and aqueous fraction of *N. Congesta* showed a dose dependent protection of RBCs from *P. berghei* infection associated hemolysis.

### Evaluation of Curative Activity

The effects of the extract and aqueous fraction on parasitemia suppression, weight, and temperature change mean

**Table 1** Phytochemicals in the Leaves of *N. Congesta* Hydromethanolic Extract.<sup>25,28,29</sup>

Secondary Metabolites	Types of Test	Results
Saponins	Distilled water	+
Steroid	H <sub>2</sub> SO <sub>4</sub> (10 mL) + chloroform (10 mL)	+
Phenol	Ferric Chloride	+
Flavonoids	NaOH + H <sub>2</sub> SO <sub>4</sub>	-
Cardiac glycoside	Glacial acetic acid + ferric chloride + concentrated H <sub>2</sub> SO <sub>4</sub>	+
Terpenoids	H <sub>2</sub> SO <sub>4</sub> (3 mL) + chloroform (2 mL)	+
Tannins	Distilled water + 1% ferric chloride	+
Alkaloid	Wagner's, Mayer's reagent	-
Anthraquinones	Benzene + 10% of ammonia hydro-oxide	-

**Notes:** + = present, - = absent.

**Table 2** Effects of *N. Congesta* Leaf Extract on Parasite Suppression and Survival Days

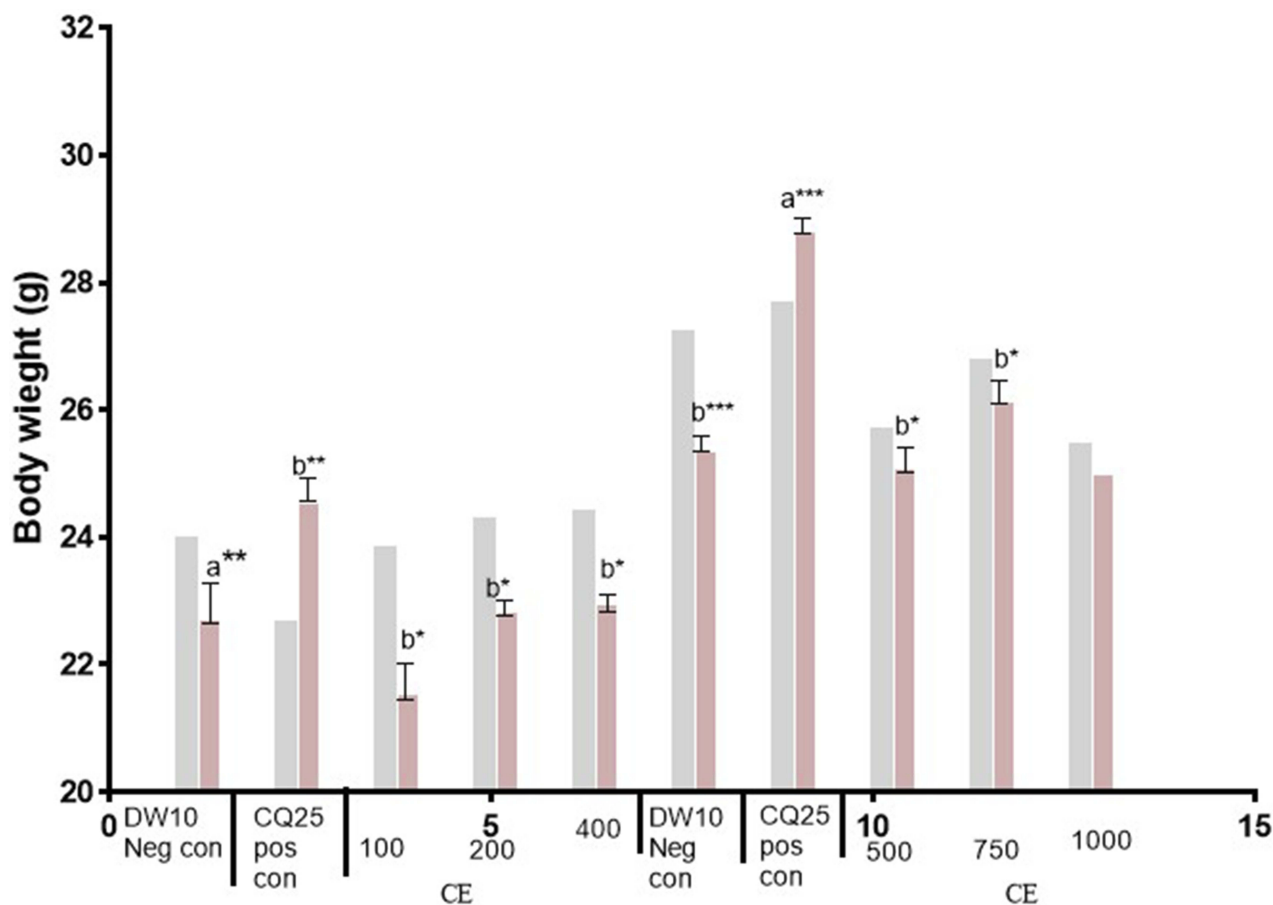
Ex	Dose (mg/kg)	Parasitemia Level	% Suppression	Survival Days
CE	100	54.8±0.081b***	2.56	7.00±0.44b***
	200	54.72±0.88b***	4.27	7.80±0.58b***
	400	54.16±1.52b***	4.98	7.40±0.50b***
DW	10 mL	56.24±2.23b***	0	5.80±0.37b***
CQ	25	0.00±0.00a	100	30.00±0.00 a***
CE	500	24.56±1.55a*b***	41.38	8.60±0.50a*b***
	750	22.86±0.55a**b***	45.44	9.00±0.70a**b***
	1,000	17.54±1.38a***b*	58.13	9.40±0.24a**b***
DW	10 mL	41.90±7.36b***	0	6.40±0.40b***
CQ	25	0.00±0.00a***	100	30.00±0.00

**Notes:** Values are presented as Mean±SEM; n=5. <sup>a</sup> compared to the negative control, <sup>b</sup> compared to the positive control. \*P<0.05, \*\*P<0.01, \*\*\*P<0.001.

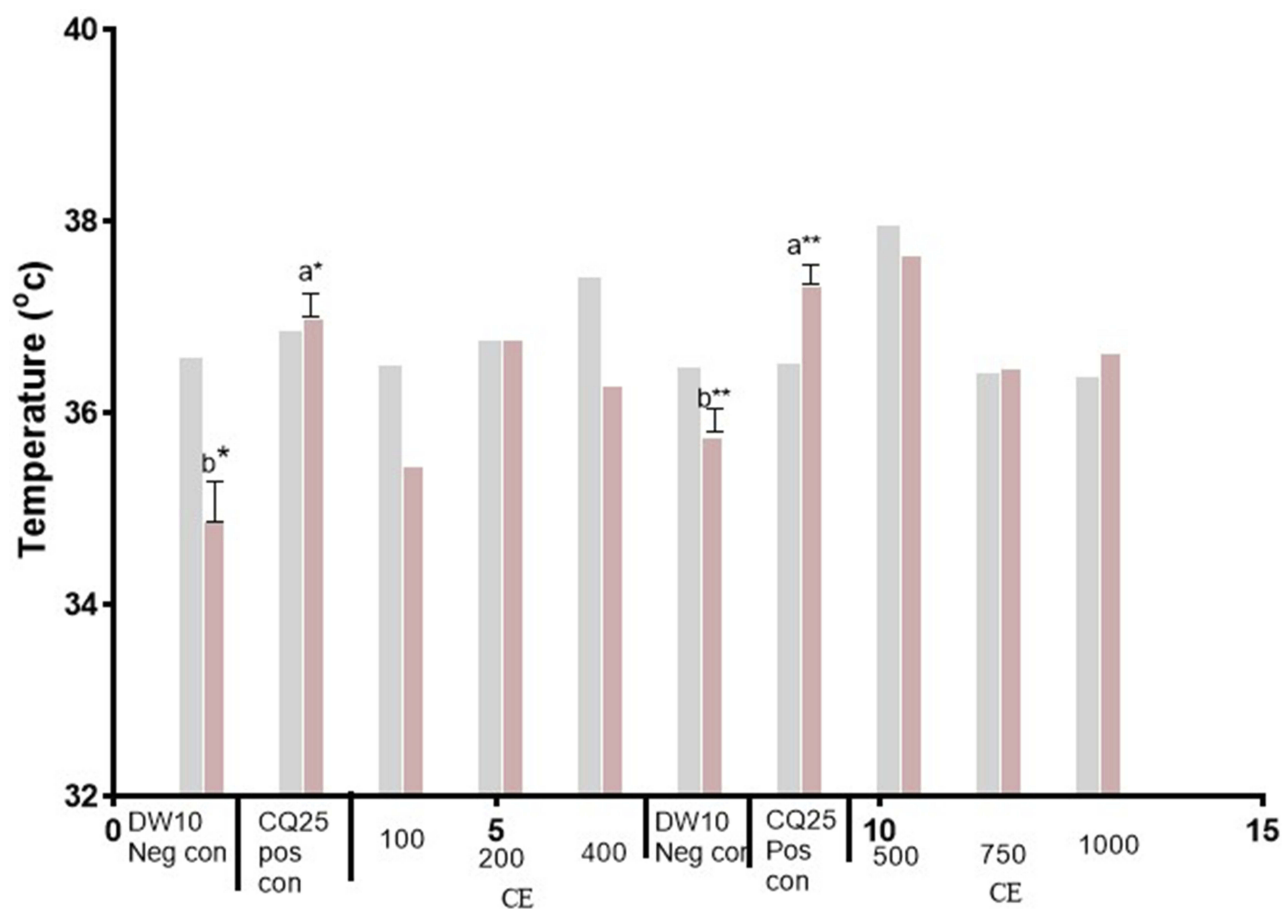
**Abbreviations:** Ex, Extract; DW, distilled water (negative control); CQ, Chloroquine (positive control); CE, crude extract.

survival time and packed cell volume are presented in Tables 5 and 6. Crude extract (1,000 mg/kg) showed significant ( $P<0.05$ ) reduction in parasitemia level at day 7

compared to the negative control. Aqueous fraction (400 mg/kg, and 600 mg/kg) showed a significant parasitemia suppression effect ( $P<0.05$ ) at days 5–7, but



**Figure 1** Body weight (D0 and D4) of *Plasmodium* infected mice treated with hydromethanolic leaf extract of *N. Congesta* in the 4-day suppressive test. Values are presented as Mean±SEM; n=5. Values are significant at  $P<0.05$ . <sup>a</sup> compared to the negative control, <sup>b</sup> compared to the positive control. \* $P<0.05$ , \*\* $P<0.01$ , \*\*\* $P<0.001$ . Legend: D0, day before infection; D4, fourth day after infection; DW10 Neg con, Distilled water 10 mL/kg Negative control; CQ25 Pos con, Chloroquine 25 mg/kg Positive control; CE, Crude Extract; 100, 100 mg/kg CE; 200, 200 mg/kg CE; 400, 400 mg/kg CE; 500, 500 mg/kg CE; 750, 750 mg/kg CE; 1,000, 1,000 mg/kg CE.



**Figure 2** Body temperature (D0 and D4) of *Plasmodium* infected mice treated with hydromethanolic leaf extract of *N. congesta* in the 4-day suppressive test. Values are presented as Mean±SEM; n=5. Values are significant at  $P<0.05$ . <sup>a</sup> compared to the negative control, <sup>b</sup> compared to positive control. \* $P<0.05$ , \*\* $P<0.01$ . ■=D0, ■=D4.

**Abbreviations:** DW10 Neg con, Distilled water 10 mL/kg Negative control; CQ25 Pos con, Chloroquine 25 mg/kg Positive control; CE, Crude Extract; 100, 100 mg/kg CE; 200, 200 mg/kg CE; 400, 400 mg/kg CE; 500, 500 mg/kg CE; 750, 750 mg/kg CE; 1,000, 1,000 mg/kg CE; D0, day before infection; D4, fourth day after infection.

extract and aqueous fraction failed to cure infected experimental animals. Only the chloroquine treated group showed total eradication of the established *P. berghei* infection on day 6.

The apparent survival time difference was not observed between the extract (500 and 750 mg/kg) and aqueous fraction (200 and 400 mg/kg) treated groups and vehicle treated groups. However, at the maximum experimental doses, both the extract and fraction showed a significantly ( $P<0.05$ ) longer survival days as compared to the negative control. Neither crude extract nor aqueous fraction exhibited significant protection of packed cell volume reduction as compared to the negative control (Table 5).

Extract and aqueous fraction failed to protect body weight and temperature loss at all test concentrations, while chloroquine (positive control) showed significant ( $P<0.001$ ) body weight and temperature loss protection as compared to the negative control (Table 6).

## Discussion

Management of malaria is a big challenge due to the increasing number of cases of resistance to the majority of currently available drugs, even to new artemisinin-based combination therapy (ACT) drugs, the increasing insecticide resistance of mosquitoes, and the unavailability of effective vaccines and other novel interventions to control the cases.<sup>35,36</sup> *P. falciparum* malaria is a life threatening disease if not treated promptly with effective anti-malarial medicines; it can rapidly progress to severe illness and death.<sup>8,37</sup> So, there is an urgent need to find new alternative drugs, which have novel modes of action. Therefore, we made an effort to assess antimalarial activity of the leaves of *N. congesta* crude extract and solvent fractions in *P. berghei* infected mice models.

Plants are the main sources of antiprotozoal compounds for the development of drugs against many tropical diseases including malaria.<sup>38</sup> In Ethiopian society, *N.*

**Table 3** Effects of n-Hexane, Chloroform, and Aqueous Fractions on Parasite Suppression and Survival Days

Fr	Dose (mg/kg)	Parasitemia Level	% Suppression	Survival Days
AQf	100	30.56±1.85b***	16.27	7.60±0.40b***
DW	10	36.50±4.01b***	0	6.60±0.24b***
CQ	25	0.00±0.00a***	100	30.00±0.00a***
AQf	200	33.28±3.87b***	20.57	9.40±0.60a*b***
	400	32.12±3.138b***	23.34	9.40±0.92a*b**
	600	23.10±1.79a*b**	44.88	9.80±0.58a**b***
DW	10	41.90±7.36b***	0	6.40±0.40b***
CQ	25	0.00±0.00a***	100	30.00a***
Hf	100	59.40±1.50b***	0	6.60±0.24b***
	200	61.80±6.30b***	0	7.40±0.24b***
	400	68.20±5.56b***	0	6.80±0.24b***
Cf	100	63.2±2.90b***	0	7.80±0.58b***
	200	47.30±3.49b***	15	8.20±0.20a*b***
	400	64.00±6.26b***	0	7.60±0.50b***
3% T80	10	55.60±3.97b***	0	6.00±0.54b***
CQ	25	0	100	30.00±0.00a***

**Notes:** Values are presented as Mean±SEM; n=5. Values are significant at  $P<0.05$ , <sup>a</sup>compared to the negative control, <sup>b</sup> compared to the positive control. \* $P<0.05$ , \*\* $P<0.01$ , \*\*\* $P<0.001$ .

**Abbreviations:** Fr, Fraction; DW, distilled water; 3%T80 (negative control), 3% Tween 80; CQ (positive control), chloroquine; AQf, aqueous fraction; Cf, chloroform fraction; Hf, n-hexane fraction.

*congestais* widely used in the treatment of various health problems like malaria, inflammation, rheumatism, joint swelling, tonsillitis, and common cold<sup>10,18,20,21</sup> and some of *Nuxia* species have been reported to have in vitro antiparasitodal activity<sup>(39,41)</sup>. The plant's traditional use and phylogenetic information are powerful clues to select a plant for bioactivity screening.<sup>32,42</sup> By considering the in vitro antimalarial report on other *Nuxia* species and traditional antimalarial use of *N. congesta*, the in vivo activities of the leaf of *N. congesta* 80% methanol extract and solvent fractions have been evaluated.

Yield of crude extract of *N. congesta* leaves (29%) was nearly similar to that of methanolic extracts of a related plant, *N. floribunda* (20%).<sup>43</sup> This high yield of hydro-methanolic crude extract of the experimental plant could be due to the high efficiency of methanol in cell walls degradation and hydromethanol could partition a wider polarity range of compounds than pure methanol. In the same case, a higher amount of aqueous fraction was

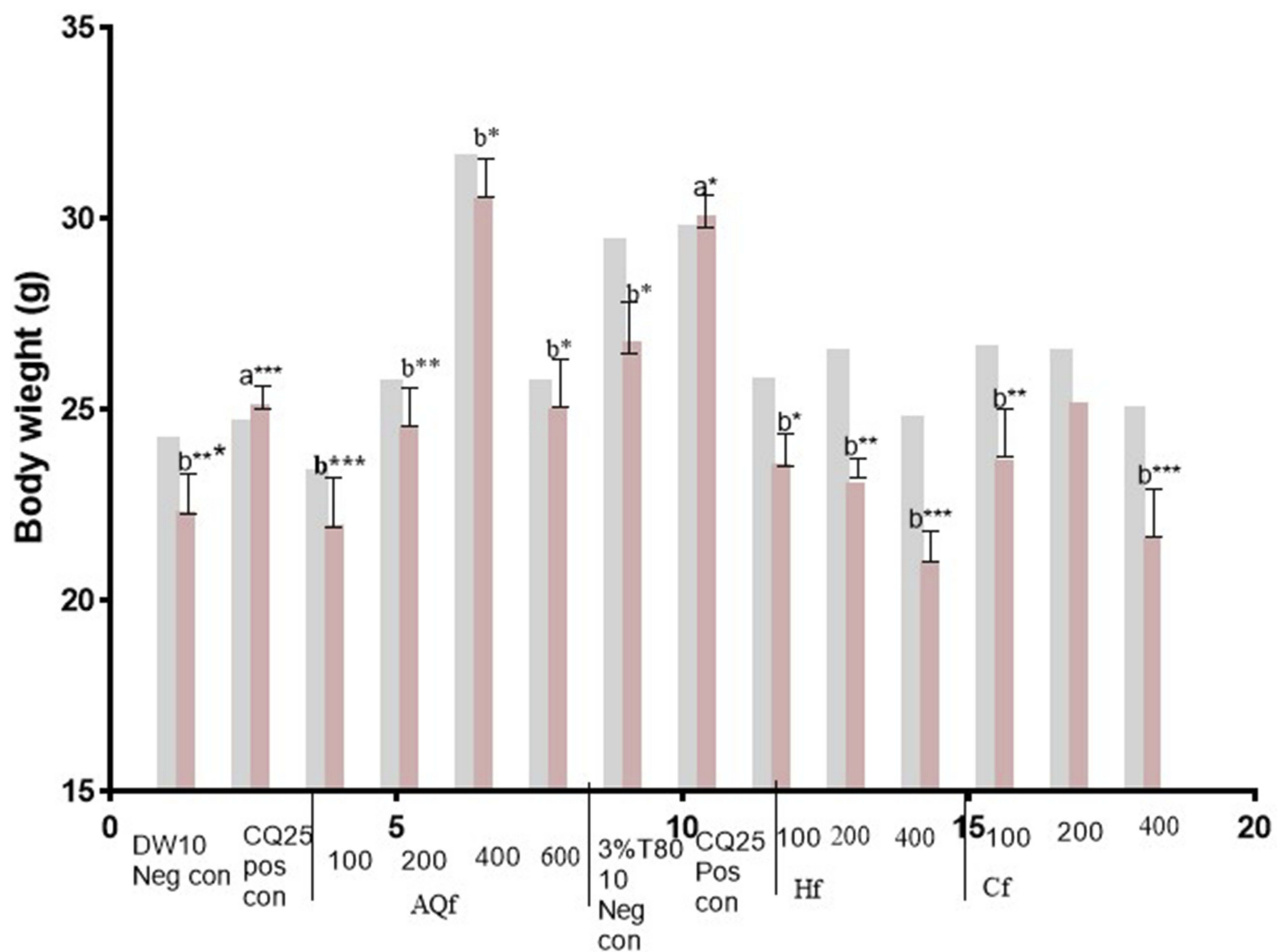
obtained than other fractions. This might be due to the presence of a high concentration of polar compounds in the leaves of the *N. congesta*.<sup>15,25</sup>

As revealed by phytochemical analysis, secondary metabolites (tannins, saponins, terpenoids, and phenols) that have been implicated in antiparasitodal activity of several other plants were also detected in the hydromethanolic leaf extract of *N. congesta*. This is in agreement with data reported by Adugna et al<sup>44</sup> in which these and other secondary metabolites have been identified. Most of these metabolites act on biomembranes; for example, saponins have amphiphilic nature, which can complex with cholesterol in biomembranes with their lipophilic moiety and bind to surface glycoproteins and glycolipids. Most of the terpenoids are lipophilic in nature and they readily interact with the lipophilic inner core of the membrane bi-layer. Phenolic compounds, which are present in most herbal medicines, carry one or several hydroxyl functional groups, which can form several hydrogen bonds with electronegative atoms (O, N) of membrane protein. These lead to alteration of protein structure, impairment of its function, hole and pore formation, which leads to leakage of cell constituents.<sup>26,45,46</sup>

Regarding acute oral toxicity of the experimental plant, a hydromethanolic leaf extract did not cause any overt morbidity and mortality in the experimental mice at 2,000 mg/kg and 5,000 mg/kg oral administration. This showed that a lethal dose of 50% is greater than 5,000 mg/kg, which attests that plant products are frequently considered to be safe and have fewer adverse effects than synthetic ones.<sup>37</sup>

The in vivo antiparasitodal activities of *N. Congesta* crude extract and solvent fractions were investigated by evaluating the chemosuppression during early infection, while crude extract and aqueous fraction were used to evaluate the curative activity of the plant during established infection (Rane's test). In both suppressive and curative tests, determination of percent parasitemia suppression and improved survival days are the most widely used parameters for antiparasitodal activity screening.<sup>31,32</sup> Since 500, 750, and 1,000 mg/kg crude extract and 200, 400, and 600 mg/kg aqueous fraction showed significant parasite suppression, they were further evaluated in curative test.<sup>47</sup> In the 4-day chemosuppressive and Rane's test, the highest parasitemia suppression (100%) was recorded in the group treated with positive control (chloroquine), while the lowest parasitemia suppression (0%) was recorded in the group treated with the negative control,





**Figure 3** Body weight (D0 and D4) of *Plasmodium* infected mice treated with hydromethanolic leaf extracts/solvent fractions of *N. congesta* in the 4-day suppressive test. Values are significant at  $P < 0.05$ , <sup>a</sup> compared to the negative control, <sup>b</sup> compared to the positive control. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ . □ = D0, ■ = D4.

**Abbreviations:** DW10 Neg con, Distilled Water 10 mL/kg Negative control; CQ25 Pos con, Chloroquine 25 mg/kg Positive control; 3%T80 (negative control), 3%Tween 80; AQf, Aqueous fraction; Hf, n-hexane fraction; Cf, Chloroform fraction; 100, 100 mg/kg AQf/Hf/Cf; 200, 200 mg/kg AQf/Hf/Cf; 400, 400 mg/kg AQf/Hf/Cf; D0, day before infection; D4, fourth day after infection.

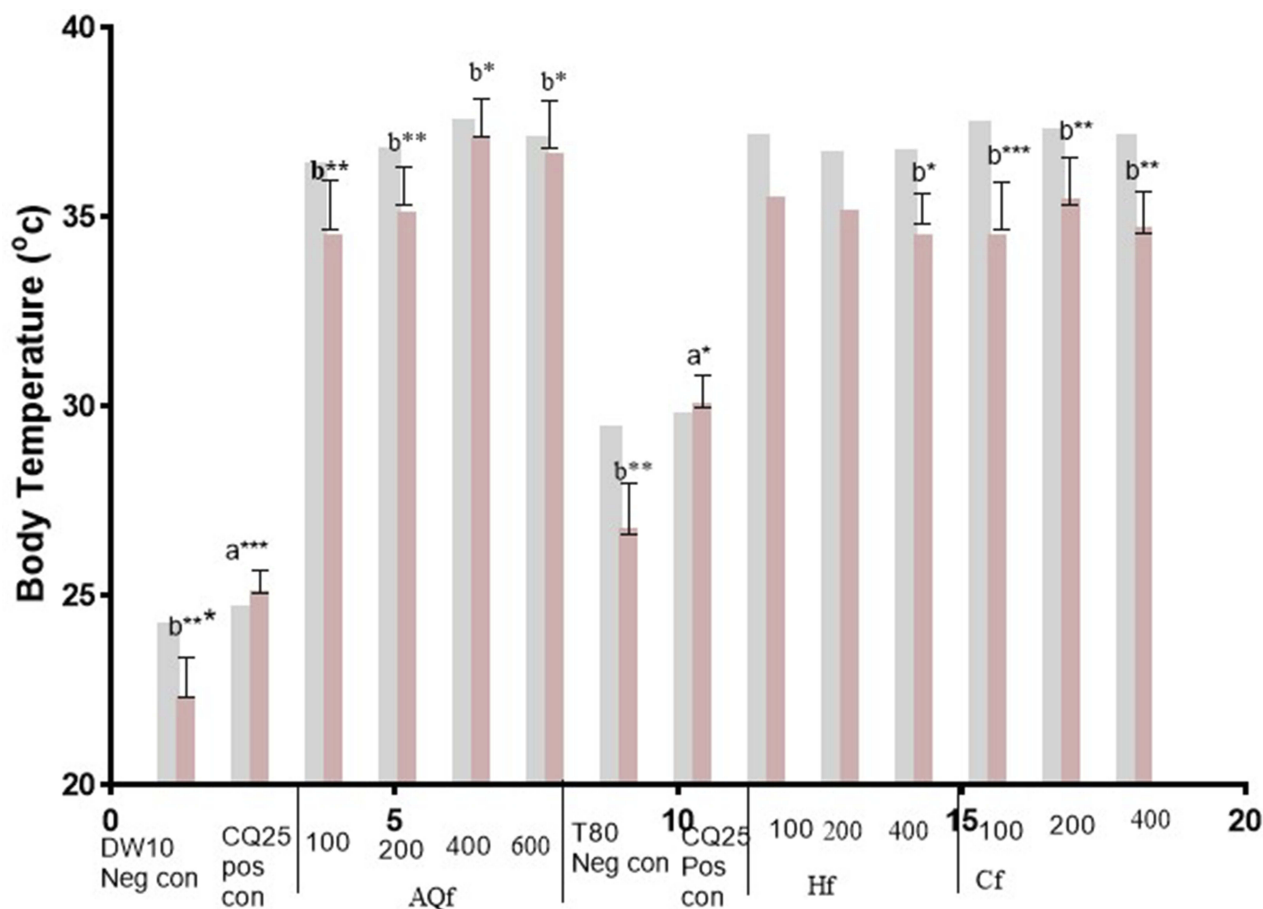
distilled water. *N. congesta* hydromethanolic extract exhibited dose dependent parasitemia suppression and prolonged the survival days of *P. berghei* infected mice, the highest activity recorded at the maximum dose administered. This is comparable with the activities of the previous reports exhibited by *Polyalthia longifolia*, *Trichilia heudelotii*, and *melia azedarach*.<sup>12,27</sup> Parasitemia level in all extract and aqueous fraction treated groups was less than the group treated with negative controls. This revealed that extract and its aqueous fraction had a suppressive effect on the multiplication and erythrocyte infectivity of *P. berghei* parasites in mice.

The hydromethanolic leaf extract of *N. congesta* suppressed parasitemia by 58.3%, the highest chemosuppression recorded from all the fractions evaluated in the current study. However, at lower concentrations (100,

200, 400 mg/kg), it exhibited insignificant activity as compared to the negative control. This could be attributable to the presence of an insufficient concentration of active principles at lower doses of the extract.<sup>15,32</sup> The curative activities were significant at 1,000 mg/kg crude extract at day 7 and 400 and 600 mg/kg of aqueous fraction at days 5–7.

Crude extract contains a collection of medicinal compounds and activities of one compound may mask the other or may be potentiated,<sup>32,46</sup> hence, fractionation may help to eliminate potentially toxic, inactivating, and/or potentiating compounds.<sup>42</sup>

Aqueous fraction demonstrated dose dependant higher activity in blood parasitemia suppression against *P. berghei* than other fractions. The relatively higher activity of the aqueous fraction could be due to secondary metabolites present in



**Figure 4** Body temperature (D0 and D4) of *Plasmodium* infected mice treated with solvent fractions of *N. congesta* in the 4-day suppressive test. Values are significant at  $P < 0.05$ , <sup>a</sup> compared to the negative control, <sup>b</sup> compared to the positive control. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ . □ = D0, ■ = D4.

**Abbreviations:** DW10 Neg con, Distilled Water 10 mL/kg Negative control; CQ25 Pos con, Chloroquine 25 mg/kg Positive control; 3%T80 (negative control), 3%Tween 80; AQf, Aqueous fraction; Hf, n-hexane fraction; Cf, Chloroform fraction; 100, 100 mg/kg AQf/Hf/Cf; 200, 200 mg/kg AQf/Hf/Cf; 400, 400 mg/kg AQf/Hf/Cf; D0, day before infection; D4, fourth day after infection.

crude extract which might be partitioned in the aqueous fraction.<sup>32,42</sup> A report from the aqueous fraction of *Nuxia* species confirmed the presence of iridoid glycosides (mono terpenes),<sup>19</sup> which are water soluble<sup>48,49</sup> and have antibacterial, anti-inflammatory,<sup>50</sup> antioxidant, immune modulating, and pain killing properties.<sup>49</sup> These effects are highly associated with antimalarial activities.<sup>34,42,47</sup>

The MST is a very important parameter to evaluate the antimalarial activity of plant extracts.<sup>31</sup> The aqueous fraction prolonged the survival time of *P. berghei* infected mice dose dependently. This might be due to the presence of secondary metabolites that prevent the overall pathologic effect of the parasite in the infected mice, like anti-inflammatory, antioxidant effects in addition to suppression of the parasite.<sup>51</sup>

N-hexane fraction did not show any parasitemia inhibition or prolongation of survival days in all dose-treated groups. On

the other hand, at higher doses of the fraction the parasitemia level was increased. This could be due to loss of active constituents during fractionation<sup>32,42</sup> or the constituents partitioned in this fraction may be immunosuppressants which can lower the immune system.<sup>17</sup>

The chloroform fraction did not exhibit dose dependent chemosuppressive activity, efficacy diminished as the dose increased. This inconsistent effect might be attributed to constituents in the fraction which may follow different kinetics.<sup>52,53</sup>

In vivo percent mean suppression  $\leq 90\%$  with respect to negative control usually indicates the test compound is active.<sup>46</sup> Accordingly, crude extract at 1,000 mg/kg (58.33%), aqueous fraction at 600 mg/kg (44.88%), and chloroform fraction at 200 mg/kg (15%) could be considered to be active against *P. berghei*. This activity

**Table 4** Activity of the Leaves of *N. Congesta* Extract and Aqueous Fraction on Paced Cell Volume in Suppressive Test

Ex/Fr	Dose (mg/kg)	PCV		
		D0	D4	Change
AQf	100	55.60±1.20	44.40±0.97	-11.20±0.58b***
DW	10 mL	57.20±0.86	43.60±1.02	-13.60±0.70b***
CQ	25	57.20±0.85	57.70±0.85	0.50±0.60a***
AQf	200	56.20±0.80	51.20±2.08	-8.83±3.80
	400	54.40±1.77	50.20±1.59	-7.65±1.68
	600	56.60±1.77	52.40±1.20	-7.29±1.35
CE	500	57.80±3.27	53.20±1.59	-6.97±5.10
	750	55.40±0.67	51.60±1.43	-6.80±2.87
	1000	55.00±2.30	52.40±1.80	-4.43±3.03
DW	10 mL	52.00±1.04	43.40±2.15	-16.61±3.52b**
CQ	25	53.40±2.69	52.60±2.37	-1.39±0.62a**

**Notes:** Values are presented as Mean±SEM. <sup>a</sup> compared to the negative control, <sup>b</sup> compared to the positive control. Values are significant at  $P<0.05$ . \*\* $P<0.01$ , \*\*\* $P<0.001$ .

**Abbreviations:** Ex/Fr, Extract/Fraction; CE, Crude Extract; DW, Distilled Water (negative control); CQ (positive control), Chloroquine; D0, Day before infection; D4, Fourth day after infection; AQF, aqueous fraction; PCV, Packed Cell Volume.

could be attributed to a single or a combination of its secondary metabolites.

Temperature and weight reduction are the hallmark of malaria infection in rodent models, indicating that an effective antimalarial agent should prevent temperature and body weight reduction in *Plasmodium* infected rodents. The dose dependent suppressive activities of the leaves of *N. congesta* crude extract and water fraction proposes that crude extract and water fraction do not have any toxic effects in experimental mice at the doses evaluated. Our acute oral toxicity study result also indicated that the plant extract was found to be safe at the maximum dose tested (5,000 mg/kg).<sup>38</sup> However, tremendous body weight loss has been observed in mice treated with 400 mg/kg of n-hexane and chloroform fractions; this could be attributable to the feed intake depressing effect of metabolites and interfere with the protein digestion by saponins and tannins.<sup>54</sup>

## Conclusion

The methanolic extract and aqueous fraction of *N. congesta* leaves have in vivo anti-malarial activities. The antimalarial activity and lack of acute toxicity is suggested

**Table 5** Evaluation of Curative Activity of *N. Congesta* Leaf Extract and Aqueous Fraction

Ex/Fr	Dose (mg/kg)	Parasitemia Level							PCV				
		D4	D5	D6	D7	%Supp	Survival Days	D3	D7	Change			
CE	500	18.50±3.61	60.25±6.27b***	84.33±3.28b***	85.00±0.0b***	10.05	7.00±0.00b***	52.25±1.37	43.00±2.00	-18.13±1.47b***			
	750	12.64±0.61	54.20±7.22b***	72.25±8.71b***	74.66±1.86	20.99	7.60±0.50b***	51.80±0.66	44.33±0.88	-14.73±0.40b***			
	1,000	15.27±0.67	36.55±3.47b***	60.00±10.63	51.50±9.5 a#b***	45	8.70±0.62a#b***	50.75±1.25	44.00±1.00	-13.09±2.74b***			
AQf	200	12.37±0.28	46.82±5.85b***	65.50±8.42	75.87±5.96 b***	19.70	7.25±0.25b***	51.75±0.85	41.75±0.85	-19.32±0.77b***			
	400	10.18±1.44	40.60±3.41b***	56.25±5.28a#b***	64.50±3.50a#b***	31.17	7.60±0.60b***	52.20±0.66	43.50±0.50	-16.34±0.96b***			
	600	11.70±0.47	40.16±2.45b***	46.10±2.10a#b***	58.50±5.48a#b***	38	8.75±0.94a#b***	52.75±0.75	45.00±1.15	-14.03±0.83b***			
CQ	25	14.25±4.66	2.50±0.64a***	0	0	100	30.00±0.00a***	51.00±0.70	56.50±1.04	10.91±3.45a***			
DW	10	17.40±2.18	57.80±3.36b***	88.00±1.00	94.50±1.50	0	6.60±0.24b***	51.40±0.67	40.00±1.0	-23.01±3.40b***			

**Notes:** Values are presented as mean±SEM; n=5. <sup>a</sup> compared to negative control, <sup>b</sup> compared to positive control. Values are significant at  $P<0.05$ , \* $P<0.05$ , \*\* $P<0.01$ , \*\*\* $P<0.001$ .

**Abbreviations:** Ex/Fr, Extract/Fraction; DW, Distilled Water (negative control); CQ, Chloroquine (positive control); CE, Crude Extract; AQF, Aqueous fraction; PCV, Packed Cell Volume; D3, Day 3 (after infection); D4, Day 4 (post-treatment); D5, Day 5 (post-treatment); D6, Day 6 (post-treatment); D7, Day 7 (post-treatment); %Supp, % parasitemia suppression.

**Table 6** Effect of the Leaves of *N. Congesta* Extract and Aqueous Fraction on Body Weight and Temperature in Curative Test

Ex/Fr	Dose (mg/kg)	Body Weight (g)		Body Temperature (°C)		% Weight Change	% Temp Change
		D3	D7	D3	D7		
CE	500	24.65±0.75	22.15±0.65	34.87±0.20	33.40±0.23	-7.29±0.77b***	-4.38±0.23b***
	750	24.80±0.37	23.33±0.26	34.72±0.14	33.35±0.64	-6.04±0.44b***	-3.81±0.32b***
	1,000	24.15±0.42	22.85±0.42	35.02±0.47	33.82±0.12	-5.38±0.36b***	-3.42±0.34b***
AQf	200	25.37±0.49	23.60±0.58	34.77±0.15	33.40±0.14	-6.95±0.95b***	-3.95±0.28b***
	400	25.00±0.52	22.90±0.50	34.64±0.15	33.52±0.14	-6.53±0.31b***	-2.82±0.54b***
	600	25.05±0.33	24.00±0.55	34.57±0.17	33.66±0.24	-5.12±1.23b***	-2.22±0.51b***
CQ	25	23.95±0.25	25.42±0.49	34.87±0.75	36.50±0.25	6.18±1.12a***	4.65±0.57a***
DW	10 mL	23.84±0.26	22.00±0.40	34.86±0.14	33.10±0.10	-8.13±0.43b***	-5.14±1.10b***

**Notes:** Values are presented as mean±SEM; n=5. Values are significant at P<0.05. <sup>a</sup> compared to the negative control, <sup>b</sup> compared to the positive control. \*\*\*P<0.001.

**Abbreviations:** Ex/Fr: Extract/Fraction; DW (negative control), Distilled Water; CQ (positive control), Chloroquine; CE, Crude Extract; AQf, Aqueous fraction; D3, Day 3 (3 days after infection and before treatment); D7, Day 7 (7 days post-treatment); % weight change, percent of body weight change from D0 to D4; % temp change, percent of temperature change from D0 to D4.

to uphold the earlier claims made by the Ethiopian traditional practitioners.

## Abbreviations

ACT, Artemisinin-based Combination Therapy; ANOVA, Analysis of Variance; OECD, Organization for Economic Cooperation and Development; IP, Intraperitoneal; MST, Mean Survival Time; PBS, Phosphate Buffered Saline; PCV, Packed cell volume; PRBCs, Packed red blood cells; RBC, red blood cell; SEM, Standard Error of Mean; SPSS, Statistical Package for the Social Sciences; WHO, World Health Organization.

## Ethics Approval

All the experiments were conducted in accordance with internationally accepted laboratory animal use, care, and guidelines. Ethical approval for the study was obtained from the Department of Pharmacology/University of Gondar Ethics and Research Committee, Gondar-Ethiopia (SOP4/78/09).

## Data Sharing Statment

Almost all the materials and data of our study are included in the manuscript, some of the material and data will be available to other researchers upon reasonable request.

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## Author Contributions

1. All authors have made substantial contributions in the conception and design, acquisition of data, analysis, and interpretation of data;
2. All authors have made substantial contributions in drafting the article, and revising it critically for important intellectual content;
3. All authors approved the final version the manuscript to be published;
4. All authors have agreed on the work and have given final approval of the version to be published.

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