

In Vivo and In Vitro Anti-trypanosomal Evaluation of Crude Methanolic Extracts of *Crotalaria albicaulis* and *Cistanche phelypaea* against *Trypanosoma evansi*

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Abstract

Camel trypanosomiasis is a major disease of economic importance in Ethiopian Somali Regional State, Ethiopia. Local people use the leaves of *Crotalaria albicaulis* and *Cistanche phelypaea* to treat this disease and complement the absence or the limitations of existing anti-trypanosomal chemotherapeutic agents. Thus, the aim of this study was to evaluate the *in vivo* and *in vitro* anti-trypanosomal activity of the methanolic crude extracts of leaves of *Crotalaria albicaulis* and *Cistanche phelypaea* against *Trypanosoma evansi* isolate. The fresh leaves of both plants were extracted by percolation technique using methanol. For the *in vivo* assay, mice infected with *T. evansi* were administered intraperitoneally daily for 7 days with crude extracts at doses of 100, 200 and 400 mg/kg body weight. *In vitro* trypanocidal activity of these extracts was tested in triplicate in 96 well micro titer plates. Diminazene aceturate and dimethyl sulfoxide were used as positive and negative controls, respectively. At 4.0 mg/ml concentration the methanolic crude extract of *C. albicaulis* completely ceased motility of the parasite after 40 minutes. The plant also significantly ($p < 0.05$) prolonged infection period *in vitro* infectivity test. Moreover, at 400 mg/kg dose, the crude extracts of both plants exhibited a mild *in vivo* anti-trypanosomal activity against *T. evansi*. This study established that leaves of *C. albicaulis* has a mild *in vivo* and *in vitro* anti-trypanosomal activity and can be considered as a potential source of new drugs for the treatment of Camel trypanosomiasis.

Keywords: *Crotalaria albicaulis*; *Cistanche phelypaea*; Anti-trypanosomal activity; *Trypanosoma evansi*

Introduction

T. evansi is a species belonging to the subgenus Trypanozoon and is the causative agent of Camel trypanosomiasis (surra). This disease is the most important cause of economic losses in Camel rearing areas, causing significant morbidity of up to 30% and mortality of around 3%. [1,2]. Camels that came into contact with tsetse flies acquired infections, and when such Camels moved to non-tsetse areas, transmission was spread by other haematophagous flies. The seasonal abundance of biting flies such as Tabanus, and other flies capable of mechanical transmission of *T. evansi* was reported to be as one of the contributing factor for the seasonal increment in the prevalence of this disease. Clinically it is manifested by weakness, lethargy, tachycardia, fever, pale mucosa, subcutaneous edema in brisket and eyelids, nasal and ocular discharges, abortion in pregnant Camel and weight loss [3].

Control of surra requires effort by integrating the treatment of infected animals with effective drugs with reducing blood sucking flies by regular insecticide treatment. Treatment with trypanocidal drugs is the usual method for the control of *T. evansi* and quinapyramine, diminazene-di-aceturate, phenazone and procaine hydrochloride have been used in Camels, and only recently melarsomine (cymelarsen) was introduced for the treatment of surra in Camels because of the problem of drug resistance [4,5]. However, most of these drugs are not curative and toxic for Camels [6,7]. Hence, considering the economic burden of this disease, the cost and the rapid emergence of resistance against clinically available anti-trypanosomal drugs, there is a great need for the development of less toxic and less expensive agents.

Plants have been used traditionally to treat different animal ailments in different parts of the world. Moreover, natural products have also been the main source for discovery and development of lead compounds against different human and animal diseases [8]. Quassinoids, isolated from traditionally used plant *Brucea javanica*, demonstrated *in vitro* anti-trypanosomiasis activity with IC_{50} value ranging from 2.9 to 17.8 μ M [9].

Local people in Somali regional state of Ethiopia have used the leaf of *Crotalaria albicaulis* and *Cistanche phelypaea* for the treatment of Camel trypanosomiasis for centuries [10]. Hence, this work was conducted to evaluate the *in vivo* and *in vitro* anti-trypanosomal effects of crude methanol leaf extracts of *C. albicaulis* and *C. phelypaea* against *T. evansi* field isolate.

Methods

Reference drug and chemicals

The commercial trypanocidal drug used was diminazene aceturate (Veriben[®]).

Collection of plants

The leaves of *C. albicaulis* and *C. phelypaea* were collected around Jigjiga, Somali regional state of Ethiopia, 599 km away from Addis Ababa. Identification and authentication of the plant materials was made at National Herbarium, Department of Biology, Addis Ababa University, where a voucher specimen (YT001) was deposited for future reference.

Preparation of plant material

The collected leaves of *C. albicaulis* and *C. phelypaea* were shade dried and powdered. The powdered plant materials were then extracted by percolation for 72 hours at room temperature with methanol (400

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ml). The extract was then filtered with Whatman filter paper (No.1) and concentrated under low pressure to dryness using Rotary evaporator at 37-40°C and finally freeze-dried.

Experimental animals and test organisms

Swiss albino mice of either sex, weighing 25-35 g, obtained from Ethiopian Health and Nutrition Institute (EPHI), were used in the present study. The animals were housed in polypropylene cages and maintained on standard pelleted diet and water *ad libitum*.

The animals were acclimatized for a period of 7 days at room temperature (23-25°C) with a relative humidity of 60-65% before starting the assay.

T. evansi parasite was originally isolated from naturally infected Camels around Jigjiga, Eastern part of Ethiopia. The organisms were maintained by serial passages in mice until required as described in the previous study [11,12]. The isolates were first inoculated heavily to donor mice which were then subjected to cardiac puncture and blood was collected with an ethylene-diamine-tetraacetic acid (EDTA) coated syringe and immediately was diluted with phosphate buffered saline (PBS). For the subsequent *in vivo* test, about 0.2 ml of the blood/PBS that contains approximately 10⁵ parasites was intraperitoneally injected into mice. Infection and parasitemia were monitored every morning by microscopic examination of blood samples taken from the tail of the infected mice using Herbert and Lumsden method [13].

Biological assays

Acute toxicity study: All animals were deprived of food for 8 hours prior to commencing the experiment. The crude extracts of *C. albicaulis* and *C. phelypaea* were then administered (orally) separately at a dose of 2000 mg/kg to two female swiss albino mice [14]. After two days, the same dose of both extracts was administered orally to eight female mice, four mice for each extract, increasing the number of treatment animals to ten (five for each extracts). The third group of five female mice, negative control group, was administered with equal volume of saline. All the mice were observed critically during the first 4 hours, periodically for the first 24 hours and once a day for 14 days. During this period gross changes such as loss of appetite and activities related to motor-muscle coordination and central and autonomic nervous system were observed.

***In vitro* anti-trypanosomal activity test:** Assessment of *in vitro* trypanocidal activity was performed in triplicate in 96 well micro titer plates. Infected blood was collected using EDTA coated syringe by cardiac puncturing donor mice at peak parasitaemia. Stock solution of each of the test substances was first prepared in 10% DMSO in PBS to produce extract solution of concentrations of 20.0, 10.0, 2.0 and 0.5 mg/mL to produce effective test concentrations of 4.0, 2.0, 0.4 and 0.1 mg/mL, respectively. Blood (20 µL) containing about 20-25 parasites per field was obtained and mixed with 5 µL of each concentration of both extracts and a similar concentration of DMSO and diminazene aceturate was included to serve as negative and positive controls, respectively [15,16].

After 5 min incubation in closed microtiter plates maintained at 37°C, about 20 µL of test mixtures was placed on separate microscope slides and covered with 7 × 22 mm cover slips and the parasites were observed every 5 min for death/motility for a total duration of one hour using X400 objective.

***In vivo* infectivity test:** To further confirm the *in vitro* effect of the test substances, the mixtures were inoculated into apparently healthy mice which were then monitored for development of infection (parasitemia) Healthy mice (5 animals per dose) received each mixture intraperitoneally (after two hours incubation) and were observed for 30 days [17,18].

***In vivo* anti-trypanosomal activity test:** Healthy mice were randomly divided in to eight groups of six mice each. All groups were then infected intraperitoneally with 0.2 ml of infected blood, diluted with PBS, containing approximately 10⁵ trypanosome. Crude extracts of both plants at three different doses, 100, 200, 400 mg/kg of body weight, were dissolved in 10% DMSO. With the establishment of parasitaemia, three concentrations of each extracts were administered orally for the total of six groups for seven consecutive days. Positive control group received 28 mg/kg (intraperitoneal single dose) of the standard drug diminazene aceturate dissolved and reconstituted in distilled water. And finally the negative control group was administered with the solvent used for reconstitution, DMSO.

Blood samples were collected from the tail end of each mouse to check the parasitemia and for determination of Packed Cell Volume (PCV). Parasitaemia was monitored on every other day through microscopic examination of blood obtained from the tail [13]. For the assessment of anti-trypanosomal effect of the test compounds, the level of parasitaemia (expressed as log of absolute number of parasites per milliliter of blood) in the treated animals was compared with those of the control animals [19]. A special scale, the micro-hematocrit reader was used to obtain the PCV percentage and the reading recorded [17]. PCV was monitored on day of parasite inoculation, treatment initiation, and at 7th and 14th day post-treatment initiation.

Statistical analysis: The data was analyzed using Statistical Package for Social Science (SPSS), version 20.0. The data obtained from the study were summarized and expressed as mean ± standard error of mean (SEM). One-way ANOVA followed by Tukey's multiple comparison tests were carried out to compare the results obtained from different groups and to determine statistical significance. P values less than 0.05 were considered statistically significant.

Results

In vitro anti-trypanosomal activity

Methanolic extract of *C. albicaulis* and *C. phelypaea* were analyzed for their *in vitro* trypanocidal activity against *T. evansi* motility at effective concentration of 4.0, 2.0, 0.4 and 0.1 mg/ml. The crude leaf extract of *C. albicaulis*, at a concentration of 4.0 mg/mL, completely immobilized or killed the parasites within 40 min of incubation, while the same concentration of diminazene aceturate immobilized or eliminated the parasite within 10 min (Table 1). Furthermore, except for the lowest concentration tested (0.1 mg/ml), all other concentration of diminazene aceturate eliminated trypanosomal motility within an hour. The remaining groups including the groups treated with the extract of *C. phelypaea*, on the other hand, showed the presence of very active parasites for more than an hour.

In vivo infectivity tests

Results obtained from the *in vivo* infectivity study revealed two of the mice which received mixtures containing 4.0 mg/mL of *C. albicaulis*.

Test substance	Time (min) at which motility was ceased or reduced				Parasite motility
	4.0 mg/ml	2.0 mg/ml	0.4 mg/ml	0.1 mg/ml	
<i>C. albicaulis</i>	40	60	>60	>60	++
<i>C. phelypaea</i>	NE	NE	NE	NE	NE
DA	10	20	40	>60	+++
10% DMSO	NE	NE	NE	NE	NE

+++ : Drastically reduced motility, ++ : Moderately reduced motility, DA: Diminazene aceturate, DMSO: Dimethyl sulfoxide, NE: No noticeable effect on motility even after 60 minutes

Table 1: Time (minutes) after which motility of *Trypanosoma evansi* parasites ceased after incubation with different concentrations of test substances.

Mixture (Inoculum)	Number of mice which developed infection	Infection interval in days (Mean±SEM)
4.0 mg/ml CA+0.02 mL Ib	2/5	17.50 ± 0.45a
2.0 mg/ml CA+0.02 mL Ib	4/5	13.75 ± 0.35 a
0.4 mg/ml CA+0.02 mL Ib	5/5	12.25 ± 0.48
0.1 mg/ml CA+0.02 mL Ib	5/5	11.20 ± 0.4
4.0 mg/ml CP+0.02 mL Ib	5/5	11.20 ± 0.25
2.0 mg/ml CP+0.02 mL Ib	5/5	11.30 ± 0.58
0.4 mg/ml CP+0.02 mL Ib	5/5	11.40 ± 0.24
0.1 mg/ml CP+0.02 mL Ib	5/5	11.80 ± 0.66
4.0 mg/ml DA+0.02 mL Ib	0/5	Ni
2.0 mg/ml DA+0.02 mL Ib	0/5	Ni
0.4 mg/ml DA+0.02 mL Ib	1/5	16.33 ± 0.33a
0.1 mg/ml DA+0.02 mL Ib	3/5	14.50 ± 0.80a
0.1 ml 10% DMSO+0.02 mL Ib	5/5	11.25 ± 0.36

N=6, Ni=no infection developed in the observation period, CA=*Crotalaria albicaulis*, CP= *Cistanche phelypaea*; DA=diminazeneaceturate; DMSO=dimethylsulfoxide; Ib=infected blood; a=significant compared to negative control.

Table 2: Duration (days) after which parasitemia developed in groups of mice inoculated with a mixture of test substances and *Trypanosoma evansi* infected blood.

On the other hand one and three of the mice received diminazene acetate at a concentration of 0.4 mg/mL and 0.1 mg/mL, respectively become parasitemic after 30 days post-inoculation (Table 2).

C. albicaulis at a concentration of 4.0 mg/ml significantly ($P<0.05$) prolonged the infection period compared to the negative control (Table 2). However, all the mice treated with a negative control (10% DMSO and parasite incubated) and at all doses of *C. phelypaea* were found to be parasitemic after 11 days of monitoring.

***In vivo* anti-trypanosomal activity test**

5.3.1 Effect of test substances on parasitemia: The parasitemia of infected but untreated and treated groups are shown in Table 3. In all the groups, parasites were first detected 14 days post infection and the mean parasite count was 7.40 ± 0.03 . There were fluctuations in the level of parasitemia among the treated groups, but the level relatively remained low particularly in groups treated with extract of *C. albicaulis*. This was particularly apparent between 6-12 days post-treatment when maximum level of parasitemia was observed in infected untreated control group.

It was observed that there were mild reductions in the activity of trypanosomes and also the level of parasitemia in 400 mg/kg of leaf extract of *C. albicaulis* treated groups (Table 3). Besides, the groups treated with *C. phelypaea* showed a high level of parasitemia throughout the observed period with no statistically significant difference ($p>0.05$) from that obtained in the infected untreated group. On the other hand, diminazene acetate at a dose of 28 mg/kg had a total clearance of parasitemia from the 2nd day post-treatment without relapse of infection

until 10 days after treatment. The methanol leaf extract of *C. albicaulis* at 100 mg/kg, 200 mg/kg and 400 mg/kg had kept parasitemia at a significantly low level on day 6, 8, 10, 12 and 14 ($p<0.05$) as compared with the negative control.

Effect of crude extracts on packed cell volume: Table 4 shows that *T. evansi* caused a significant PCV reduction ($p<0.05$) in infected but untreated mice as the average value was found to drop from 46.00 ± 0.79 on the day of parasite inoculation to 40.10 ± 1.10 on 14 days post treatment. All groups treated with the extract exhibited a statistically significant ($p<0.05$) prevention of reduction of PCV on 14th day post treatment compared to the negative control. However, mice treated with 100 and 200 mg/kg doses of *C. phelypaea* had a reduced PCV values, though statistically indifferent ($p>0.05$) from those treated with other groups.

Effect of crude extracts on body weight: Treatment with crude extracts of *C. albicaulis* (400 mg/kg and 200 mg/kg) significantly ($p<0.05$) prevented loss of body weight associated with parasitemia compared to the negative control group (Figure 1a and 1b). Throughout the monitoring period, considerable body weight improvement was observed by 28 mg/kg diminazene acetate relative to the pre-treatment value.

Effect on mean survival time: All doses of *C. albicaulis* and the highest (400 mg/kg) dose level of *C. phelypaea* have significantly prolonged survival time after infection compared to the negative control group (28.8 ± 0.34) (Figure 2). On the other hand, mice treated with diminazene acetate had the highest mean survival time of 40.20 ± 0.31 days.

Rx	Dose (mg/kg)	Parasitemia (log no)/ml							
		D0	D2	D4	D6	D8	D10	D12	D14
DMSO	-	7.43 ± 0.23	8.1 ± 0.21	8.6 ± 0.25	8.65 ± 0.19	8.71 ± 1.02	9.36 ± 0.89	8.94 ± 1.33	8.72 ± 2.32
CP	100	7.34 ± 0.31	8.09 ± 0.30	7.72 ± 1.12	7.70 ± 0.81	7.81 ± 1.21	7.91 ± 0.09	8.08 ± 1.08	8.26 ± 1.34
	200	7.53 ± 0.29	7.67 ± 0.25	7.61 ± 0.97	7.42 ± 0.97 ^a	7.52 ± 1.25	7.81 ± 1.23	7.84 ± 1.16	8.42 ± 0.78
	400	7.46 ± 2.34	5.86 ± 1.03	6.9 ± 0.99	6.92 ± 2.01	6.84 ± 4.01	6.56 ± 2.23	6.8 ± 1.97	6.90 ± 0.87
CA	100	7.90 ± 0.54	8.14 ± 0.58	7.58 ± 0.67	7.63 ± 0.67 ^a	7.82 ± 0.39	7.92 ± 0.19 ^a	7.92 ± 0.58	7.46 ± 1.12
	200	7.37 ± 0.19	7.8 ± 0.34	4.71 ± 0.93	4.82 ± 0.63 ^a	6.02 ± 0.27 ^a	5.70 ± 1.61 ^a	5.83 ± 1.36 ^a	6.01 ± 0.53
	400	7.46 ± 2.34	5.86 ± 1.03 ^a	6.9 ± 0.99 ^a	5.92 ± 2.01 ^a	5.84 ± 4.01 ^a	5.56 ± 2.23 ^{ab}	6.8 ± 1.97 ^{ab}	5.44 ± 0.87 ^{ab}
DA	28	7.41 ± 0.34	7.67 ± 0.28	00 ± 0.00 ^{abc}	00 ± 0.00 ^{abc}	00 ± 0.00 ^{abc}	00 ± 0.00 ^{abc}	2.96 ± 1.19 ^{abc}	3.22 ± 1.20 ^{ab}

Values are Mean ± SEM, N=6, D=day, D0=the day treatment commenced. DA=Diminazene aceturate, CA=*Crotalaria albicaulis*, CP=*Cistanche phelypaea* and DMSO; dimethylsulfoxide; a=significant compared to negative control, b=compared to CP100 mg/kg, c=compared to CA100 mg/kg; Rx=treatment.

Table 3: Effect of leaf methanol extracts of *Crotalaria albicaulis* and *Cistanche phelypaea* on parasitemia levels of *Trypanosoma evansi* infected mice.

Treatment	Dose (mg/kg)	PCV/Day of treatment		
		PCV0	PCV7	PCV14
CA	100	47.10 ± 0.68	42.40 ± 1.07	45.10 ± 0.91 ^a
	200	48.30 ± 1.11	44.00 ± 1.50	48.20 ± 0.46 ^a
	400	47.00 ± 1.21	43.60 ± 1.41	47.60 ± 1.44 ^a
CP	100	50.20 ± 1.20	44.20 ± 1.65	47.20 ± 0.73
	200	47.90 ± 0.60	44.30 ± 0.80	46.10 ± 0.87
	400	47.10 ± 0.57	45.90 ± 1.45	47.30 ± 0.73 ^a
DA	28	50.10 ± 0.33	48.60 ± 0.57 ^a	50.60 ± 0.43 ^a
DMSO	-	46.00 ± 0.79	42.10 ± 1.18	40.10 ± 1.10

Values are Mean ± SEM, N=6, D=day, D0=the day treatment commenced; CA=*Crotalaria albicaulis*, CP=*Cistanche phelypaea*, DA=diminazene aceturate, DMSO=dimethylsulfoxide, a=significant compared to negative control.

Table 4: Effect of *Crotalaria albicaulis* and *Cistanche phelypaea* leaf methanol extract on packed cell volume (PCV) of *Trypanosoma evansi* infected mice.

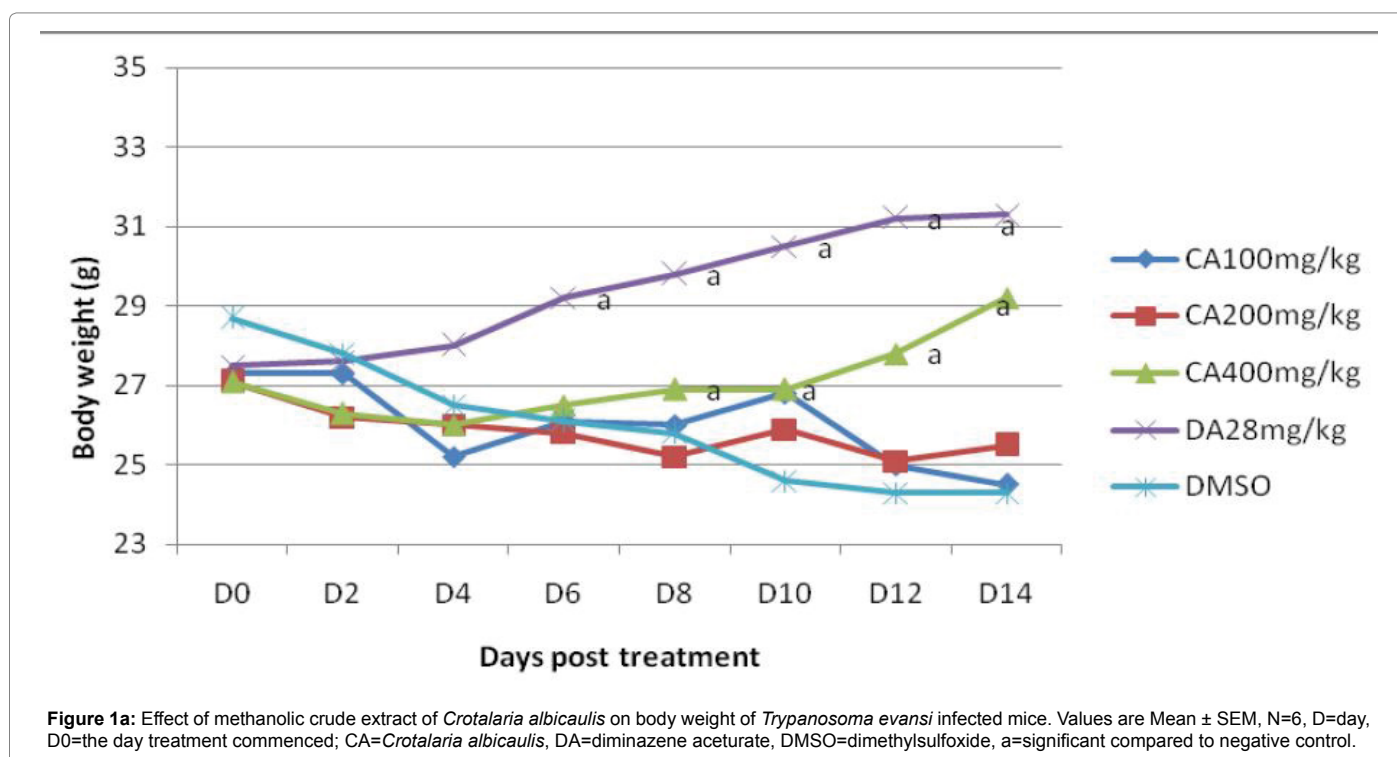


Figure 1a: Effect of methanolic crude extract of *Crotalaria albicaulis* on body weight of *Trypanosoma evansi* infected mice. Values are Mean ± SEM, N=6, D=day, D0=the day treatment commenced; CA=*Crotalaria albicaulis*, DA=diminazene aceturate, DMSO=dimethylsulfoxide, a=significant compared to negative control.

dose showed parasite motility inhibition comparable to the activity of *Artemisia abyssinica* (2 mg/ml) against *T. congolense* [21]. On the other hand, the parasite motility inhibition effect of *C. albicaulis*, recorded in this study, is better than a previous study done on aqueous and methanol leaf extract of *Verbascum sinaiticum* in which the 4 mg/ml dose require 60 minutes to cease motility [22]. The *in vitro* anti-trypanosomal activity of *C. albicaulis* observed in this study might be attributed to either the individual class of compounds present in each herb or to the synergistic effect that each class of compounds exerts to give the observed biological activity. Thus, further in-depth phytochemical and pharmacological investigations should be conducted to determine this issue.

In this study, higher dose of *C. phelypaea* showed significant ($p < 0.05$) reduction in percentage parasitemia as compared to negative control group (Table 3). Especially, 400 mg/kg of *C. albicaulis* group had significantly ($p < 0.05$) lower mean parasitaemia (5.44) as compared to the negative control group (8.72) on day 14. This finding is in line with previous reports on the activity of *Carrisa edulis* [23], *A. abyssinica* [24], *Adansonia digitata* [25] against different trypanosomal species. The reduction in percentage parasitemia reported in these and in the current study could be due to the effect of phytochemicals to interference with the redox balances of the parasites acting either on the respiratory chain or on the cellular defenses against oxidative stress [26,27]. Furthermore, this might also provide the scientific basis for the traditional use of *C. albicaulis* in the management of trypanosomiasis in Somali Regional State, Ethiopia [10].

Trypanosomes were not totally eliminated from the bloodstream of infected mice in all experimental groups as compared to diminazene aceturate treated control group within 11 days of treatment. This could be attributed to the pharmacokinetic inactivation of the active principles of the extracts or the release of trypanosomes from tissues during delayed or inadequate treatment [27,28] and, despite the temporary clearance of bloodstream forms of trypanosomes, relapse occurred in all mice treated with diminazene aceturate at day 12 post-treatment initiation. This is in line with other studies done on *T. congolense* [24]. This observation indicates the existence of resistance to diminazene aceturate which has been reported to be widespread in South-western part of Ethiopia [7].

Anemia is a constant feature of trypanosome infections whose severity is linked to the level of parasitemia [24,25]. In this study, all the experimental groups treated with the standard drug (diminazene aceturate) and higher doses of both extracts showed significantly ($P < 0.05$) higher PCV as compared to negative control group (Table 4). This might be due to the nature of some phytochemicals to reduce the parasite load, neutralize the toxic metabolites produced by trypanosomes and scavenge the trypanosome associated free radicals [22]. In agreement with this fact, some finding also reported the anti-trypanosomal activity of *M. balsamina* and *S. longipendunculata* and their effect on maintaining relatively high PCV value in infected mice [29-31]. The extracts to improve the PCV possibly by reducing the parasite load or inactivating the toxic metabolites produced by trypanosomes [21,31].

Body weight loss, associated with progression of *T. evansi* infection in mice, is usually due to loss of appetite [26]. In this study, however, 400 mg/kg of *C. phelypaea* and middle and higher dose of *C. albicaulis* significantly ($p < 0.05$) prevented weight loss as compared to the negative control groups. This finding was found to be in line with other similar activities which was done with different plant extracts [12,32]. This could be due to the overall effect of extracts to reduce parasitemia and maintain of PCV of *T. evansi* infected mice.

Conclusion

The present study revealed that the crude methanolic extract of the leaf of *C. albicaulis* possesses mild *in vitro* and *in vivo* anti-trypanosomal activities against *T. evansi* field isolate. Thus, considering the promising activity of *C. albicaulis* along with its relatively wide margin of safety, further analysis is required to identify potent biologically active chemicals.

Data Availability

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethical Approval

For *in vivo* study, all procedures complied with the Guide for the Care and Use of Laboratory Animals and OECD guideline 425 for acute toxicity. Research directorate office of Jigjiga University has approved this study.

Conflict of Interest

The authors declare that they have no conflicts of interest.

Author Contribution

Yitagesu Tewabe: Collected plant material, carried out the experimental work and drafted the manuscript, edited and proof read the manuscript before submission.

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