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Prophylactic Effects of Methanolic Extract of *Artemesla absynthuem* in *Trypanosoma congolense* Infected Mice

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Abstract: An experiment was conducted to determine the *in vivo* prophylactic anti-typanosomal effect of methanol extracts of the aerial parts of *Artemisia absinthium* against *Trypanosoma congolense*. Thirty-six Swiss white male mice aged between 6-8 weeks were divided into six experimental groups each consisting of six animals. Methanol extract of the plant was prepared and *T. congolense* was isolated from cattle at Didessa valley (western Ethiopia) and propagated on mice. All groups received $1x10^5$/ml of trypanosomes in 0.2 ml of blood /PBS via intra peritoneal route of inoculation. The methanol extract of *A. absinthium* was given orally to three groups, at doses of 400, 800 and 1200 mg/kg body weight for 7 consecutive days before infection. One group remained as infected non treated control and the other group received Isometamidium chloride thus served as treated control. The last remaining group represented non infected and non treated (distilled water only). The prophylactic effect of the extract on levels of parasitaemia, body weight, PCV and mice survival was monitored for 70 days. No parasitaemia was detected by wet film examination up to 21 days post infection with *T. congolense* in mice treated with methanol extracts of *A. absinthium* and Isometamidium chloride. The time variation in parasite appearance in plant extract treated mice was dose dependent hence in mice infected with 800mg/kg and 1200mg/kg of the plant extract, the level of parasitaemia was very low and comparable with those group infected and treated with Isometamidium chloride. Meanwhile, the level of parasitaemia was significantly lower ($p<0.01$) in these mice compared to non-treated infected control. Groups receiving methanol extracts of *A. absinthium* and Isometamidium chloride had significantly higher body weights than the untreated infected group ($P<0.01$). Significant higher PCV and mice survival time were observed in groups treated with methanol extracts of the plant and Isometamidium chloride compared to the untreated infected control ($P<0.01$). It can be concluded that the plant extract had a good prophylactic potential against pathogenic trypanosomes (*T. congolense*) of livestock importance by reducing the levels of parasitaemia, maintaining good PCV, body weight and survival of infected animals.

Key words: *Artemisia absinthium* · Isometamidium Chloride · Mice · Parasitaemia · PCV · Survival Time · *Trypanosoma congolense* · Body Weight

INTRODUCTION

Trypanosomosis is a protozoan disease of both animals and human caused by different species of the genus *Trypanosoma*. Trypanosomes are transmitted by the bite of infected tsetse flies (*Glossina* species). Biting flies such as *Tabanus*, *Haematopota* and *Stomoxys* species can transmit blood steam trypomastigotes to mammalian hosts and this has enabled some species of pathogenic trypanosomes to spread widely beyond Africa into Asia and Latin America. In Ethiopia, the most important trypanosome species in economic terms are *Trypanosoma congolense, T. vivax* and *T. brucei* [1, 2]. A report by Abebe and Jobre [3] indicated prevalence of 58.5%, 31.27% and 3.5% by *T. congolense*, *T. vivax* and *T. brucei*, respectively in cattle from tsetse-infested areas of

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southwest Ethiopia. In tsetse-free areas, 8.71% trypanosome prevalence was recorded in the highlands, out of which 99% was due to *T. vivax* [3]. Trypanosomosis has direct impact on livestock productivity, reducing meat and milk off take by 20%, calving rate by 20%, increase calf mortality by 20%, decreases both lamping and kidding rates in sheep and goat and livestock management especially the number of livestock kept by farmers, the breed and species composition of the livestock herd, the way the livestock are grazed, cost of trypanocidal drugs and cost of insecticides [4]. It also has direct impacts on human settlement in a considerable part of sub-Saharan Africa including Ethiopia [5].

The control of trypanosomes mainly relies on chemotherapy and vector control. The vector control is either by ground or Arial spray of insecticides. However, because of high cost and environmental consideration, the use of insecticide spraying becomes no more a method of choice to carry out. Currently, other alternative vector control approaches are being practiced. Targets and traps have been used extensively in agricultural settings [6] and considerable success has been achieved by directly applying insecticides (pour-on) on animals [7]. The sterile insect technique (SIT), which was applied in large scale tsetse eradication programs in some parts of Africa including Burkina Faso [8], Northern Nigeria [9], Tanzania and Zanzibar. In Ethiopia, similar vector control strategies including fly traps, targets and pour-on insecticide applications are being practiced in different parts of tsetse infested areas. Moreover, an SIT project is being coordinated by the Ethiopian science and Technology Ministry in the Southern Nations and peoples Administrative Region [10, 11].

The use of trypanocidal drugs that targets the trypanosomes in the host is one of second major approach in the control of trypanosomosis. Three antitrypanosomal drugs, Isometamidium chloride, Homidium (bromide and chloride) and Diminazene aceturate are drugs that have been on the market for over 50 years [12]. At present, the most widely used trypanocidal drugs for *T. congolense* and *T. vivax* infection in Ethiopia are Isometamidium and Diminazene aceturate. However these drugs have limitations like toxicity, drug resistance and high cost [13, 14]. Due to these limitations there are approaches towards the search of new drugs especially from plant origin. Previously trails were made on extracts of *Commiphora kerstingii* on *T. brucei brucei* [15], *Azadiracta indica* on *T. b. rhodesiense* [16] and *Neurolaena lobata* on *T. cruzi* [17]. However, no work on trypanosome species such as *T. congolense* of livestock importance has been reported until very recently. This study however did not consider different doses of the extracts and their prophylactic ability if given before the establishment of infection.

Therefore, this study was undertaken to investigate the prophylactic effect of *Artemisia absinthium* methanol extract on the level of parasitemia, PCV, body weight and survival of mice infected with *Trypanosoma congolense* following treatment with different doses of the extract.

MATERIALS AND METHODS

Experimental Mice: Swiss white male mice aged between 6-8 weeks, weighing 30-50 g body obtained from the Bishoftu National Veterinary Institute were used. The mice were kept in a room where the temperature was maintained through ventilation under a 12 h light and 12 h dark cycles. They were provided with a commercial pellet ration and water *ad-libitum* throughout the experimental period including the 15 days of acclimatization in the experimental room. The 36 mice were divided into six experimental groups: NIC (none infected control, treated with distilled water), INC (infected with *T. congolense* isolates and non-treated control), IMT (infected and treated with a single dose of Isometamidium chloride at the rate of 4 mg/kg). The other three groups received 400, 800 and 1200 mg/kg of methanolic extract of *Arthemisia absinthium* as prophylaxis for seven days, respectively (Table 1). On the eighth day all except the negative controls (NIC and INC) were infected with 1 $x10^5$ parasites in 0.2 ml blood/PBS solution intra-peritonially [18]. All animals were handled by respecting standard protocols in accordance with the good laboratory practice regulations. The study was conducted by using *Trypanosoma congolense* isolated from upper Diddesa valley of southwestern Ethiopia. During the course of the experiment, the survival rate was measured by recording the number of days the mice has lived with or without treatment, from the start of the trial (Day 0) to the end of experiment (Day 70).

Plant Material Collection and Extraction: *Arthemisia absinthium* was used for this experiment. Areal part of *A. absinthium* was collected from the field around Addis Ababa. The collected plants of *A. absinthium* were washed under running tap water to remove dust and any other foreign materials and left to drain off. The plants were then spread on laboratory benches and left to air dry for four weeks. The leaves were powdered using mortar

Table 1: Experimental design of infection and treatment schedule

Groups	Infection dose	Treatment
NIC	Non infected	Distilled water (for 7 days)
INC	1 x 10^5/ml	Non treated infected control
IMT	1 x 10^5 /ml	Isometamidium chloride 4mg/kg (single dose)
AP 400 mg/kg	1 x 10^5/ml	Methanol extract of *A. absinthium* (for 7 days)
AP 800 mg/kg	1x 10^5/ml	Methanol extract of *A. absinthium* (for 7 days)
AP 1200 mg/kg	1 x 10^5/ml	Methanol extract of *A. absinthium* (for 7 days)

and pistil. The powdered material of the plant was sieved and taken for extraction. The extraction was done at Hawassa University of Agriculture, department of phyochemistry according to the method employed by Emmert *et al*. [19].

Preparation of Methanol Extract of the Plant: The powdered plant material was extracted using the maceration method. In maceration method of extraction the powdered plant material is placed in stoppered container with the solvent and allowed to stand at room temperature for a period of at least three days with frequent agitation until the soluble matter has dissolved. The mixture then is strained, the marc (the damp solid material) is pressed and the combined liquids are clarified by vacuum suction filtration using Whatmann 42 filter paper. The marc is then transferred to small bottles, sealed and dried in a lyophilizer overnight.

***In vivo* Toxicity Test:** Before the beginning of the experimental treatment with plant extract three mice were orally drenched with 400mg/kg and 800mg/kg (experimental doses) and 2000mg/kg of the crude extract respectively for seven days for sub acute toxicity test [20]. Changes in behavior, clinical symptoms and signs of pain and stress were observed for 10-15 minutes following administration of the test drug for signs of acute toxicity, including the hypotensive response.

Measurements

Level of Parasitaemia, PCV and Body Weight: The level of parasitaemia was measured from the blood collected from the tail every other day for 28 days and every week from day 28 onwards until the end of the experiment (D70) by the 'rapid matching' method [21]. The number of parasites counted was then converted to logarithmic values to the base 10. The values obtained were taken as the number of parasites per milliliter of blood [21]. When there was no parasite in 20 fields the sample was taken as negative.

Packed cell volume (PCV) is the percentage of red blood cell (RBC) obtained by centrifuging whole blood. EDTA coated capillary tubes were used to collect blood from the tail vein of mice every other day for 28 days after infection and every week from day 28 onwards (until day 70, experimental period)**.** The capillary tube was filled to ¾, sealed on one end and centrifuged in a micro-centrifuge for 5 min at 12000 rpm. PCV was measured by using haematocrit reader and values were taken as percentage of cell (RBC) volume to total volume of blood [22].

The weight of mice was measured every other day using a weighing balance. Body weight was measured every other day from day zero before infection until day 28 after infection from day 28 onwards every seven days until the end of the experiment for those survived.

Statistical Analysis: Data on parasitemia, body weight and packed cell volume were analyzed using Windows SPSS Version 15 [23]. The one-way ANOVA was used to compare results among and within groups for differences between initial and final results. All data were analyzed at a 95% confidence interval (á = 0.05). Univariate survival analysis of data using Kaplan-Meier method was done to determine the effect of plant extracts on the survival rate of infected animals. The log-rank test was used to examine the null hypothesis that the survival times were identical.

RESULTS

Toxicity Test in Uninfected Mice: Methanolic extracts of *A. absinthium,* given at doses of 400 mg/kg, 800 mg/kg and 1200 mg/kg to the uninfected mice revealed no statistically significant difference ($P<0.05$) to the levels of PCV (Fig. 1) and body weight (Fig. 2) as compared to those of water- treated mice (NIC). In all mice, PCV and body weight gradually increased during the toxicity test period and no sign of acute toxicity was observed. Thus the extract was considered to be safe to proceed with the experiment.

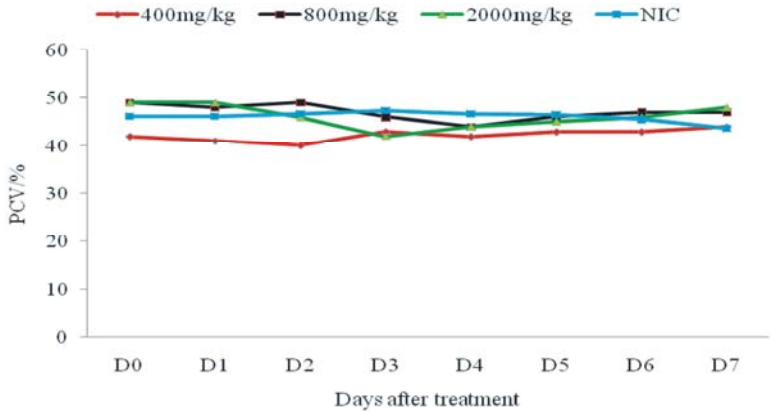

Fig. 1: Mean PCV of uninfected mice during sub acute toxicity testing (Uninfected mice were treated with either water or methanolic extracts of *A. absinthium*).

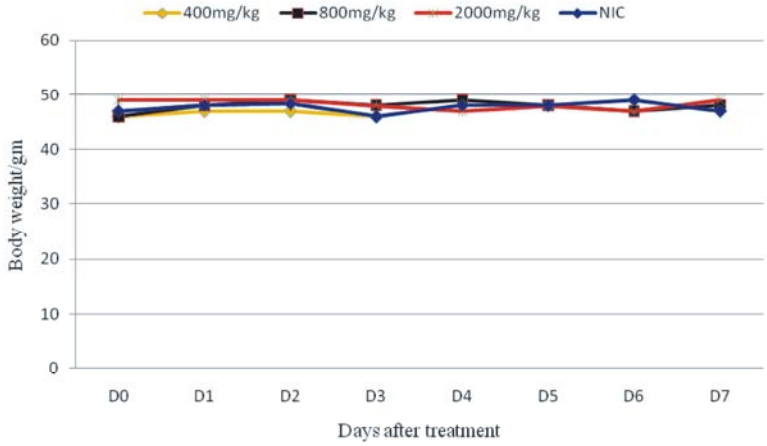

Fig. 2: Body weights of uninfected mice during sub acute toxicity testing (The mice were given either water or methanol extracts of *A. absinthium*).

Impact of Treatment on Parasitaemia: Infected-none treated mice showed the presence of parasites 21 days after infection (DAI) with all the mice being positive by day 24 (DAI). There was a variation in the first day of appearance of parasites in the blood of infected mice that were treated with the different doses of plant extracts and Isometamidium chloride. The time variation in parasite appearance in plant extract treated mice was dose dependent, i.e. on the 24th DAI for 400mg/kg and 800mg/kg plant extract and 28th day for 1200mg/kg extract group. In the Isometamidium (ISM) group first detection of parasitemia was on 42nd DAI. However, while parasitemia remained low in the ISM group and progressively increased in the INT group, variations were observed in the groups receiving different doses of the extract, being lower for 1200mg/kg and generally higher for 400mg/kg (Figure 3).

Analysis of Paired Samples t- Test for mean Parasitemia was carried out to see the impact of treatment on parasitemia. Accordingly, there was a statistically significant difference ($P<0.05$) in the mean parasitemia levels of animals found in the three doses of AP 400 mg/kg, 800 mg/kg and 1200 mg/kg and INT. However, there was no statistically significant difference ($P > 0.05$) in the mean parasitemia levels of animals found in the three doses of AP 400 mg/kg, 800 mg/kg and 1200 mg/kg and Isometamidium chloride. The mean parasitemia level of all mice in groups with methanol extracts of *A. absinthium* 400 mg/kg, 800 mg/kg and 1200 mg/kg and Isometamidium chloride showed no statistically significant difference ($P>0.05$).

Impact of Treatment on Body Weight: Body weight measurements were made every other day until the end of the experiment. The weight in the untreated infected mice

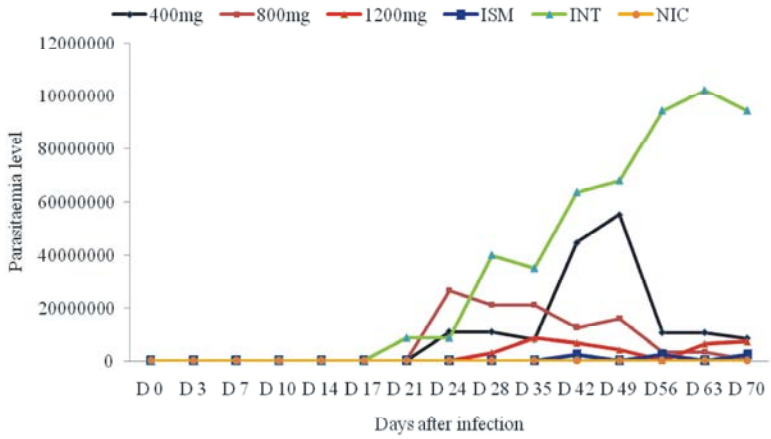

Fig. 3: Parasitaemia of *T. congolense* infection in mice treated with either water or methanol extract of *A. absinthium and* Isometamidium chloride only

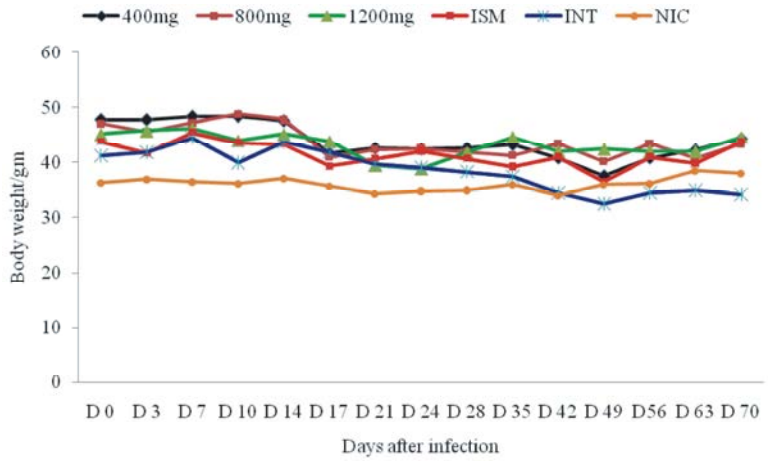

Fig. 4: Body weights of *T. congolense* infected mice treated with either water or extract of *A. absinthium* and Isometamidium chloride only

group (INT) started to decline after 14 DAI. However, all the mice in this group (INT) died few before the end of the experiment (D56 to D.63). All mice treated with methalonic extract of *A. absinthium* generally showed a gradual increase in mean body weight during the experimental period as compared to groups Isometamidium treated group (IMT) (Fig. 4). On 49th day post infection, groups receiving methanol extracts of *A. absinthium* and Isometamidium chloride had significantly higher weights ($P<0.01$) than the non-infected control (NIC) and untreated infected group (INT). Though, all the mice in groups with methanol extracts of *A. absinthium* had relatively higher body weights than the Isometamidium chloride groups, there was no statistically significant difference in body weights between these two groups ($P>0.01$).

Pair wise analysis of the impact of treatment on body weight of the mice was performed using t-Test. Accordingly, there was a statistically significant difference ($P<0.05$) in the mean body weight of animals found in the three doses of AP 400 mg/kg, 800 mg/kg and 1200 mg/kg and INT. The mean body weights of all mice in groups with methanol extracts of *A. absinthium* 400 mg/kg, 800 mg/kg and 1200 mg/kg and Isometamidium chloride showed no statistically significant difference ($P>0.05$).

Impact of treatment on PCV: There was a gradual fall in the mean PCV levels following infection in all infected mice. While the mean PCV in the untreated infected mice continued to decrease starting from 28 DAI. In the Isometamidium chloride treated group, the level of mean

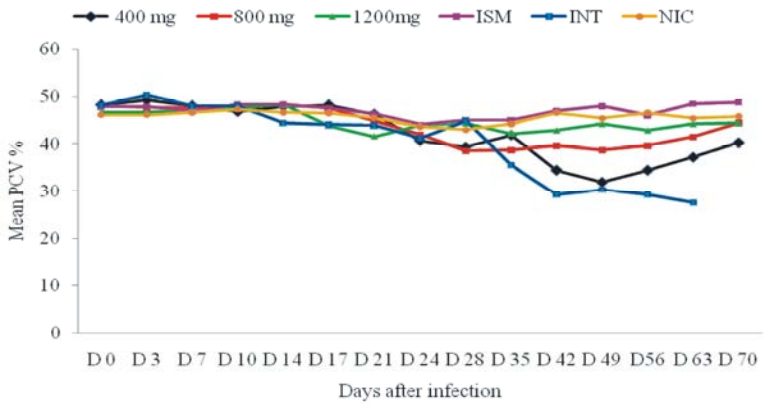

Fig. 5: Mean PCV levels in *T. congolense* infected mice treated with either water or extract of *A. absinthium* and Isometamidium chloride only

Table 2: Survival time of experimental mice

Treatment groups	No of mice survived
400 mg/kg	5/6
800 mg/kg	5/6
1200 mg/kg	6/6
ISMM	6/6
INT	0/6
NIC	6/6

PCV was consistently higher as opposed to all the remaining treatment and control groups. Methalonic of *A. absinthium* treated mice with the doses of 800 mg/kg and 1200 mg/kg particularly showed a gradual increase in mean PCV from 24 DAI until the end of the experimental (Fig. 5). All mice treated with methanol extract of *A. absinthium* with the doses of 800 mg/kg and 1200 mg/kg showed a relatively higher and increasing levels of PCV like the trends in Isometamidium chloride and NIC groups. On day 28 for INT group and 35 DAI for mice treated with 400 mg/kg *A. absinthium* observed sharp decrease in mean PCV levels compared to other experimental groups. There was no statistically significant difference (P>0.05) in mean PCV levels between groups treated with methanol extracts of *A. absinthium* (800 mg/kg and 12000 mg/kg) and Isometamidium chloride treated mice.

Pair wise analysis of the impact of treatment on mean PCV of the mice was done using t-Test. Accordingly, there was a statistically significant difference (P<0.05) in the mean PCV levels of animals treated with AP 400 mg/kg, 800 mg/kg and 1200 mg/kg and INT. The mean PCV value of all mice in groups with methanol extracts of *A. absinthium* (800 mg/kg and 1200 mg/kg) and Isometamidium chloride revealed no statistically significant difference (P>0.05).

Survival Periods Of Trypanosome Infected Mice: There were only slight differences between the three treatment groups treated with the three different doses of the extracts i.e 400 mg/kg, 800 mg/kg and 1200 mg/kg on the survival time of mice following infection. Death started to occur in INT group from 56 DAI and at the end of the experiment all mice were died (0% survived at the end of the experiment). In the group INT, the establishment of parasitaemia was faster than the arthimisia treated groups. In the Isometamidium treated group no death occurred (100% survived at the end of the experiment) with a similar trend in groups of 1200 mg/kg. However, in 400 mg/kg and 800 mg/kg groups of methanol extract of *A. absinthium* treated mice 66.67% survived until the end of the experiment (Table 2).

DISCUSSION

This experimental study was planned to see the prophylactic effect methanolic extract of *Artemisia absinthium* in three different doses against *T. congolense* isolates in experimentally infected male Swiss mice. *Artemisia absinthium* is grown especially in the northern and central parts of Ethiopia for its aroma and is widely applied in rituals [24]. The prophylactic effect of this medicinal plant extract was not yet investigated.

Death started to occur in INT group from 56 DAI and at the end of the experiment all mice were died (0% survived at the end of the experiment). In the group INT, the establishment of parasitaemia was faster than the arthimisia treated groups. In the Isometamidium treated group no death occurred (100% survived at the end of the experiment) with a similar trend in groups of 1200 mg/kg. *T. cruzi*, *T. b. brucei* and *T. b. rhodesiense* who have

shown that extracts of different plants may exhibit *in vivo* antitrypanosomal activity [25-27].

The result of the current study indicates that the antitrypanosomal prophylactic effects were clearly observed in mice obtaining 800 mg/kg and 1200 mg/kg. This effect was observed gradually, it was comparable with mice infected with *T. congolense* and treated with a single prophylactic dose of Isometamidium chloride. Prophylactic activity manifested by the methanolic extract of *A. absinthium* might be attributed to the presence in them of some phytochemicals like flavonoids, triterpenes, terpenoids, tannins alkaloids and other constituents [28]. The *in vivo* antitrypanosomal effect of the *A. absinthium* can equally be attributed to the presence of the above secondary metabolites present as reported by Mekonnen *et al.* and Nibret and Wink [29-30]. However, further study may be needed to identify the exact molecules that have played a major role in the killing and/or suppression of *T. congolense* infection in mice. It was demonstrated that extracts of the herbs of *A. absinthium* to have effect on the growth inhibition of *T. brucei* in vitro [29]. They suggested that this activity of the extracts might be attributed to the major compound camphor and two other major sesquiterpene lactones, absinthin, artabsin and glucosinolates which are known to occur in the plants.

The findings of this study clearly indicated that the plant extract (*A. absinthium)* in the three doses of methanol extract showed moderate to high *in vivo* antitrypanosomal activity. There is a slight difference in the survival of mice between the doses. Highest survival rate is observed in 1200 mg/kg. The prolongation of lives of treated animals may therefore be associated with the ability of this extract to improve the PCV possibly by reducing the parasite load or inactivating the toxic metabolites produced by trypanosomes. The consistent suppression of parasitaemia combined with prolonged survival time of mice as shown in vivo results may be linked to the ability of the extract against the parasite.

Despite the significant reduction in parasitaemia, the plant extracts did not completely clear the parasites. The appearance of the parasitaemia was also dose dependant where parasitaemia appeared progressively in mice obtained higher doses of *A. absinthium* compared to infected but not treated control ones. Several researchers made similar observations on reduction in parasitaemia and concluded that high parasite load could mask the efficacy of crude extracts [31-32]. Moreover, the crudeness of the extracts and the oral route of administration (to avoid toxicity if given through other routes) might have also reduced the availability of sufficient active ingredients. Observations with the commercial drug, Isometamidium chloride showed an initial decrease in the level of parasitaemia (wet film) to non-detectable level and later on during the experimental period, the recurrence (relapse) of parasitaemia only in few mice from the group.

The positive effect of plant extracts can further be deduced from weight measurements of the experimental animals. Animals treated with the plant extracts on average maintained their body weight post treatment while the animals in the untreated infected group showed progressive reduction in body weights. This finding was similar to studies elsewhere [33]. Moreover, mice treated with different doses of methalonic extract of *A. absinthium* generally had comparable body weights to those treated with Isomethamedium chlorid indicating that the reduction in parasitaemia due to the extracts has led to the maintenance of close to normal body weights throughout the experimental period. The lower body weight of the NIC group is probably due to the lower initial body weight of the animals which was maintained throughout the study period.

It has been shown that the measurement of anemia gives a reliable indication of the disease status and productive performance of trypanosome infected animals [34-37]. Severity of anemia usually reflects the intensity and duration of parasitaemia. Several reports [38-40] have also ascribed acute anemia in trypanosomosis to proliferating parasites. The result of this study showed that these plant extracts have comparable potential to Isometamidium chloride since they are able to control anemia, especially at the later stages of the infection, by minimizing sudden drops in PCV values. In untreated mice, the parasite count increased and the packed cell volume (PCV) decreased markedly from day to day until the death of the animals in agreement with previous studies [27-41].

Treatment with different doses of *A. absinthium* has shown extended survival of mice in the treatment groups compared with the non-treated control group. The prolongation of survival time in infected groups following oral administration of crude extract agrees with previous reports [32, 41] who have applied extracts of *Momordica balsamina* and *Hymenocardia acida* on *T. brucei*. Of special interest are the methanolic extracts of our test plants; *A. absinthium* which clearly demonstrated an interesting antitrypanosomal profile; they maintained the animals above 65 days despite the presence of parasites in circulation.

The use and misuse of drugs has contributed to the development of drug resistance in trypanosomes. An urgent need arises therefore to develop newer drugs to counter trypanocidal resistance. This study showed that methanol extract of the aerial parts of *Artemisia absinthium* have shown parasite suppressive effects on *T. congolense* infected Swiss white mice and resulted in the improvement of PCV and body weight. The extract also prolonged the lives of mice to comparable level to that of commercial drug Isometamidium chloride. From these, it can be concluded that the plants has excellent prophylactic potential against pathogenic trypanosomes (*T. congolense*) of livestock importance. Thus, in order to investigate and exploit this potential, further study is required to dissect the active components in the crude extract of this plant, standardize the dosage and mode of application and make field trial on livestock species. In this regard, the immense plant biodiversity of Ethiopia means that the medicinal plant potential is still untouched by scientific studies. Therefore, those already known plant species of medicinal importance and those yet to be identified should be given enough attention for trials on major diseases of livestock importance. The livestock rearing community has tremendous knowledge on different ethno-veterinary practices and herbal medications. This should be systematically gathered and exploited in the selection of effective plants against various diseases including trypanosomosis.

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