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In Vitro Ovicidal and Larvicidal Activity of Aqueous and Methanolic Extracts of *Ziziphus Mucronata* Barks Against *Haemonchus Contortus*

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Abstract

Haemonchus contortus is one of the most pathogenic nematode parasites in small ruminants' worldwide. Anthelmintic resistance and high cost of drugs has prompted evaluation of medicinal plant extracts which can be used as alternative drugs. The objective of this study was to evaluate *in vitro* ovicidal and larvicidal activity of aqueous and methanolic extracts of *Ziziphus mucronata* barks against *H. contortus* stages isolated from a sheep in Kenya. Barks of *Z. mucronata* were collected from Chad, air-dried, ground and extracted with methanol and distilled water. The crude extracts were qualitatively screened for phytochemicals using standard methods. The anthelmintic activities of the extracts were evaluated using the egg hatch assay and larval mortality assay. The percentage extraction yields for methanol aqueous and aqueous methanol were 4.5% and 2.6%, respectively. The phytochemicals found in both extracts were saponins, tannins, glycosides, flavonoids and steroids. The results showed that methanolic extract had a significantly ($p<0.05$) higher activity with IC_{50} value of 3.9 mg/ml as compared with aqueous extract which had IC_{50} value of 14.7 mg/ml. In larval mortality assay, the methanolic extract had significantly ($p<0.05$) higher EC_{50} (7.5 mg/ml) than that of aqueous extract (2.7 mg/ml). The effects of Albendazole on egg hatchability inhibition and larval motility was significantly ($p<0.05$) higher than that of the two extracts. All the assays showed extract concentration dependent response. In conclusion, this study has shown that *Z. mucronata* extracts have anthelmintic activity on eggs and larvae of *H. contortus* parasite. The activity could be related to the presence of phytochemicals such as saponins and tannins. The effects of larval mortality were higher compared to that of egg hatchability. Therefore, extracts from *Z. mucronata* can be developed further as novel anthelmintic drug for control of H. contortus and hence improve production of small ruminants.

Keywords: *Haemonchus contortus*; Resistance; *Ziziphus mucronata*; Phytochemical; Inhibition

Introduction

Helminths play a significant role in causing helminthosis in small ruminants leading to enormous economic losses. These helminths occur worldwide and cause losses which are associated with among others reduced milk production, lowered weights and mortality [1].

Haemonchosis is a condition caused by *Haemonchus contortus* which has been listed among the most important conditions affecting production of small ruminants especially sheep and goats in developing countries [2].

Control of these parasites in ruminants basically depends on routine use of anthelmintic drugs. However, in recent years, the efficacy of these anthelmintic have decreased due to development of resistance by parasites [3]. This has awakened the interest of herbal medicinal plants as an alternative source for control of these parasites [4,5].

Ziziphus mucronata plant, also known as buffalo thorn, is a medium-sized with spreading canopy. In Africa, extracts from the plants are used for treatment of variety of conditions including gastro-infections, schistosomiasis, boils, chronic cough, infertility, oedema, pneumonia, snake bite, toothache, venereal diseases and wounds [6-8].

A study [9] showed that extracts from *Z. mucronata* have anti-schistosomiasis properties. In Zimbabwe, the leaves and fruits are sources of feed for small ruminants [10].

The present study was carried out to evaluate *in vitro* ovicidal and larvicidal activity of aqueous and methanolic extracts of *Z. mucronata* barks against *H. contortus* egg and larval stages isolated from sheep.

Materials and Methods

Collection and preparation of plants

Ziziphus mucronata (local name in Chad: Ngohkroh) barks (500 grams) were collected from Deli location in the southern region of Chad during the month of April 2015. The plant materials were identified and authenticated by botanists from Herbal Treatment Centre in Deli, Chad.

The collected plant materials were shade dried for three weeks at ambient temperature, ground to fine powder by using pestle and mortar. The powdered bark was packaged into envelope and transferred to Kenya. Extraction has been done at Biochemistry and Chemistry Laboratories of Jomo Kenyatta University of Agriculture and Technology.

Aqueous extraction

Aqueous extraction of *Z. mucronata* barks were prepared according the procedures described by [11]. Briefly, 200 grams of fine powder were weighed and mixed with 1000 ml of distilled water in 2 Litres flask and boiled for 2 hours. It was then cooled to 40°C and the residues filtered using Whatman filter paper No 1. The extracts were dried in a freeze dryer (Christ Alpha 1-4 LD, SciQuip Ltd, Newtown UK) and stored at 4°C until required for *in vitro* anthelmintic activity described below.

Methanolic extraction

The fine ground powder of *Z. mucronata* barks were weighed, extracted with methanol and residues filtered using Whatman filter paper No.1. The extracts were concentrated using a rotary evaporator (BUCHI R-200, Labortechnik AG CH-9230 Flawil Switzerland) at 45°C and stored at 4°C until required for anthelmintic activity.

Phytochemical screening

Aqueous and methanolic extracts of *Z. mucronata* barks were subjected to phytochemical screening using standard procedure described previously [12].

In vitro anthelmintic activity

In vitro anthelmintic activity was conducted according to World Association for the Advancement of Veterinary Parasitology (WAAVP) guidelines [13] with slight modification on parasite collection, eggs preparation, egg hatch and larval mortality assays as described below:

Parasite collection and eggs preparation

Mature adult parasites of *H. contortus* were collected directly from abomasum of slaughtered sheep from a local slaughter house in Ruiru, Kenya. The worms were transported to the laboratory in Phosphate Buffer Saline (PBS, pH 7.4).

Identification and separation of adult mature female were done using a microscope using the method [14]. They were washed using PBS and crushed using pestle and mortar in 5 ml PBS to liberate eggs. McMaster slide technique, as described [15], was used to estimate the concentrations of egg in aliquots of 200 µl.

Egg hatch inhibition (EHI) assay

For each extract, a stock solution of 4 mg/ml was taken as the initial concentrations and serial dilutions of 0.0625 to 4 mg/ml prepared in phosphate buffered saline. Approximately 100 *H. contortus* eggs in 200 µl of egg suspension were pipetted into each well of 96 well microtitre plates.

A total of 200 µl of *Z. mucronata* extracts were then added into each well. Albendazole (Albendazole, Sigma-Aldrich) was used as positive control while untreated eggs in PBS were used as negative control. Three replicates for each concentration of both aqueous and methanolic extracts of *Z. mucronata* and controls were performed. The plates were labelled and incubated for 48 hours at 27°C and 70% relative humidity. After 48 hours of incubation, hatched larvae (live or dead) and unhatched eggs were counted using a compound microscope at 40X magnification with the help of a counter [16,17]. The egg-hatch inhibition rates assessed were calculated by the following formula:

$$\%\ \text{Egg hatch inhibition} = \frac{\text{Total number eggs - number hatched larvae}}{\text{total number of eggs}} \times 100$$

Larval mortality assay (LMA)

Larval mortality assay using larvae were performed according to [18]. After collection of adult parasite, the female *H. contortus* was identified and separated from male as described above. Eggs were recovered by grinding the female with pestle and mortar in 5 ml PBS. One hundred eggs in 180 µl of egg suspension were put into each well of 96 well microtitre plates. A 20 µl of nutritive media (comprising of 1 g yeast in 90 ml of normal saline and10 ml Earle's balanced salt) was added into each well. The plates were then incubated under humidified condition at ambient temperature for 48 hours. After 48 hours of incubation, 200 µl of *Z. mucronata* extracts in concentrations ranging from 0.0625 to 4.0 mg/ml were added to respective plates. Albendazole (Albendazole, Sigma-Aldrich) was used as positive control while untreated larvae in PBS were used as negative control.

There were three replicates for each extract concentration and control. The plates were further incubated for 24 hours (total of 3 days). Counting of all larvae in each well was done under an inverted microscope. The percentage of mortality of the larvae was determined using the following formula:

$$\%\ \text{Larval mortality} = \frac{\text{Number of dead larvae}}{\text{Number of larvae in culture}} \times 100$$

Statistical analysis

Mean percentage egg hatch inhibition rates and larval mortality from *Z. mucronata* extracts and Albendazole at different concentrations were compared using one-way ANOVA test at $p<0.05$ significant levels. The inhibition concentration required to inhibit 50% (IC_{50}) for ovicidal and effective

concentration (EC_{50}) values for larvicidal activity were determined using the regression line of probit according to the log10 of the extract concentration.

Results

Extraction and phytochemical screening

The per cent yield for methanol extraction was higher (4.5 ± 0.21%) than that of aqueous extraction (2.6 ± 0.5%).

The results for phytochemicals screening of *Z. mucronata* extracts are presented in Table 1. The phytochemicals present found in the extracts included saponins, tannins, glycosides, flavonoids and steroids.

Table 1 Phytochemical screening of aqueous and methanolic extracts Ziziphus mucronata. Key:'-' Absent, '+' Present.

Phytochemical	Aqueous extracts	Methanol extracts
Saponins	+	+
Tannins	+	+
Alkaloids	-	-
Glycosides	+	+
Flavonoids	+	+
Steroids	+	+

Egg-hatch inhibition (EHI) assay

Different concentrations of aqueous and methanolic extracts of *Z. mucronata* barks in concentrations ranging from 0.0625-4 mg/ml were tested for their anthelmintic activity and results presented in the **Figure 1**.

The higher drug concentration resulted in higher egg hatch inhibition compared with lower concentration suggesting a concentration dependent response.

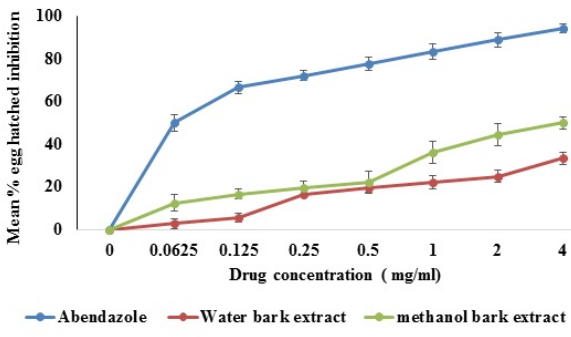

Figure 1 Mean hatch inhibition for eggs of *Haemonchus contortus* exposed to *Ziziphus mucronata* extracts of and Albendazole.

At all extracts concentrations, the egg hatch inhibition was highest in eggs exposed to Albendazole, followed by methanol and aqueous extracts. There was no significant ($p>0.05$) difference in egg hatch inhibition by aqueous and methanolic extracts of *Z. mucronata*. However, there were significant ($P<0.05$) differences in egg hatch inhibition of extracts compared to that Albendazole.

The concentration required to inhibit 50% (IC_{50}) of both extracts and Albendazole are presented in **Table 2**. Methanolic extract showed a higher activity with IC_{50} value of 3.9 mg/ml as compared with aqueous extract with IC50 value of 14.7 mg/ml.

Table 2 IC_{50} values of methanol and aqueous extracts of *Z. mucronata* barks and Albendazole exposed to eggs of *H. contortus*.

Sample type	Mean IC_{50} values (mg/ml)	Lower bound	Upper bound
Aqueous extract	14.683	6.896	55
Methanol extract	3.932	2.416	8.282
Albendazole (Positive control)	0.05	0.024	0.079
95% confidence limits for concentration (mg/ml)			

Larval mortality assay (LMA)

Results of larval mortality and concentration required to inhibit 50% of infective larvae are presented in **Figure 2** and **Table 3**, respectively.

Aqueous and methanolic extracts of *Z. mucronata* in different concentration and ratios showed variable effect on mortality of larvae of *H. contortus.*

At the maximum tested concentration of 4 mg/ml, the highest (53.3 %) larval mortality was from methanolic extracts with while the least (43.3%) was from aqueous extract.

Albendazole required a maximum concentration of 0.5 mg/ml to induce 100% larval mortality. There was a significant difference in activity among different drug/extracts concentration ($p<0.05$).

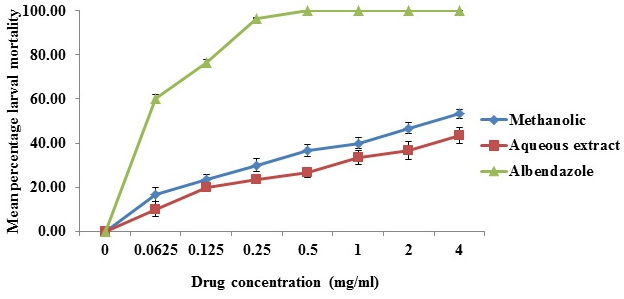

Figure 2 *In vitro* larvicidal activity of methanolic and aqueous extracts of *Ziziphus mucronata barks.*

The highest EC_{50} value against larval mortality was revealed by Albendazole (0.045 mg/ml), followed by methanolic extract (2.7 mg/ml) and least was aqueous extract (7.5 mg/ml). The EC_{50} were significantly ($p<0.05$) different from each other.

Further, the IC_{50}/EC_{50} for larval mortality was significantly ($p<0.05$) higher than that of egg hatch inhibition.

Table 3 IC_{50} of methanolic and aqueous extract of *Z. mucronata* exposed to larval stages of *H. contortus*

Sample type	IC50 values (mg/ml)	Lower Bound	Upper Bound
Water barks extract	7.516	3.638	28.032
Methanol Barks extract	2.646	1.593	5.929
Albendazole (Positive control)	0.045	0.031	0.057
95% confidence limits for concentration (mg/ml)			

Discussion

Haemonchus contortus causes significant losses in small ruminant production. Farmers in low resource settings in Africa rely on herbs to treat helminths but their efficacy has not been well evaluated. Further the phytochemicals found in these plants have also not been investigated. In the current study, the phytochemicals and efficacy of *Z. mucronata* were investigated. The phytochemical screening in the present study showed the presence of various phytochemical constituents such as flavonoids, saponins, steroids, tannins and glycosides. These significant variations in the phytochemical contents of a plant part are due to number of environmental factors as previously mentioned [19]. These phytochemicals account for their medicinal value. For instance, tannins extracted from plants have been shown to have anthelmintic, antidiarrhoeal and antimicrobial activities [20,21], while Saponins have anthelmintic, antidiarrhoeal and anticancer properties [22-24]. Glycosides and Steroids produced by the plant extracts are known for their antidiarrhoeal action [24,25], whereas Flavonoids in plants have been shown to have antidiarrhoeal and antimicrobial activities [22,26].

The current study evaluated the efficacy of bark extracts of *Z. mucronata* against egg and larval stages of *H. contortus*. The egg hatch assay was initially developed for the diagnosis of benzimidazole resistant helminths. This test has, however, been used for screening of plants compounds for their anthelmintic activity [11,27,28]. In this study, Methanolic extract showed a higher activity with IC_{50} value of 3.9 mg/ml as compared with aqueous extract with IC_{50} value of 14.7 mg/ml. This could be attributed to the difference in proportions of active components that were responsible for anthelmintic activity. A previous study [29], on aqueous extracts of *Hedera helix* showed IC_{50} value of 0.12 mg/ml respectively when tested against *H. contortus* egg. Aqueous extract of *Coriandrum sativum* had an IC_{50} of 0.12 mg/ml while the hydroalcoholic extract had an IC_{50} of 0.18 mg/ml on egg hatch inhibition test [30]. Thus, these plants had a relatively higher activity compared with *Z. mucronata* aqueous extract. Albendazole showed comparable results with that of in vitro anthelmintic activities of four Ethiopian medicinal plants against *H. contortus* which had IC_{50} value of 0.04 mg/ml [30], a value which is close to that reported in the current study. In the present study, aqueous and methanolic extracts of *Z. mucronata* in different concentration and ratios showed variable effect on mortality of larvae of *H. contortus*.

Similar to egg inhibition, the highest EC_{50} value against larval mortality was by Albendazole (EC_{50}=2.7 mg/ml) and least was aqueous extract (EC=7.5 mg/ml). Similar results by a previous study [31] showed that the EC_{50} aqueous extract of *Fumaria parviflora* against *H. contortus* was 10.23 mg/ml. Comparable results on larval mortality showed EC_{50} values of 15.14 and 12.88 mg/ml for aqueous and ethanolic extract of *Adhatoda vasica*, respectively [32]. Findings of this study showed that increasing the concentration of the plant extracts resulted in increased anthelmintic activity. A similar observation have also been made by a previous study [33] which demonstrated that the receptors get saturated with increasing concentration of active ingredient. It is likely that at higher concentration all binding receptors on the larvae were occupied thus leading to hyper-polarisation of membranes limiting excitation and impulse transmission causing flaccid paralysis of larvae muscles, a similar observation also made by previous study [34]. The present study showed that larval development inhibition by the extracts showed a better activity in contrast with egg hatch inhibition. The possible explanation could be due to the difference in structure of the egg shell and cuticle of larvae of *H. contortus* through which absorption of chemicals take place [30].

Conclusion

The findings of this study showed that, aqueous and methanolic extracts of *Z. mucronata* have a potential anthelmintic activity on eggs and larvae of *H. contortus* parasite. It is postulated that saponins and tannins identified could be responsible for the observed anthelmintic activity. Therefore, extracts from this plant can be further assessed in order to develop novel anthelmintic drug for control of *H. contortus* and other nematodes affecting livestock and man. Further studies geared towards isolation, identification, characterization and purification of bioactive compounds from this plant should be carried out in order to increase its efficacy.

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