

## IN VITRO ANTHELMINTIC ACTIVITIES OF FOUR ETHIOPIAN MEDICINAL PLANTS AGAINST *HAEMONCHUS CONTORTUS*

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### Summary

*In vitro* experiments were conducted to determine anthelmintic effects of crude aqueous and hydro-alcoholic extracts of the seeds of *Croton macrostachyus*, *Ekebergia capensis*, *Acacia nilotica* and *Terminalia schimperiana* on eggs and adult *Haemonchus contortus*. Both extract types of *C. macrostachyus* and *E. capensis* as well as aqueous extract of *A. nilotica* induced complete egg hatch inhibition at concentration less than or equal to 2 mg/ml. Based on their ED<sub>50</sub>, the three most potent plant extracts were aqueous extract of *E. capensis* (0.06mg/ml), aqueous extract of *C. macrostachyus* (0.1mg/ml) and hydro-alcoholic extract of *C. Macrostachyus* (0.32mg/ml) in decreasing order of potency. Hydro-alcoholic extract of *A. nilotica* and *T. schimperiana* inhibited hatching of eggs of *H. contortus* significantly at some concentrations tested but not in dose dependent manner. Aqueous extract of *T. schimperiana* induced no significant inhibition of egg hatching at all concentrations tested ( $p>0.05$ ). Both aqueous and hydro-alcoholic extracts of *C. macrostachyus* and *E. capensis* produced significant ( $p<0.05$ ) dose dependent mortality of adult *H. contortus*, however the activity of hydro-alcoholic extract was significantly ( $p<0.05$ ) higher than that of aqueous extract at all concentrations tested. Both aqueous and hydro-alcoholic extracts of *A. nilotica* and *T. schimperiana* have shown no statistically significant ( $p>0.05$ ) effect on survival of adult parasites at the concentrations tested, and a few mortality cases recorded were not dose dependent. The overall findings of the study revealed that extracts from *C. macrostachyus* and *E. capensis* have potential anthelmintic effect and further *in vitro* and *in vivo* evaluation is warranted to make use of these plants.

**Key words;** *Acacia nilotica*, Anthelmintic activity, *Croton macrostachyus*, *Ekebergia capensis*, *Haemonchus contortus*, *Terminalia schimperiana*

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### Introduction

Helminth infections remain a major constraint to livestock productivity across all agro-ecological zones and production systems in Africa, particularly in areas where extensive grazing is practiced [1, 2]. Today, the principal mode for control of gastrointestinal parasites is based on the commercial anthelmintics. However, wide spread increase of anthelmintic resistance, scarcity and high cost especially to farmers of low income in developing countries led to the need of other alternative helminth control methods [2, 3]. Among other alternative methods, there is considerable and expanding interest in traditional herbal dewormers in both industrialized and

developing countries [4, 5]. *Haemonchus contortus*, being a highly pathogenic and one of the most prevalent nematode parasites in Africa, it has been used by several workers to evaluate the anthelmintic effects of various medicinal plants [6, 7, 8, 9].

Ethnoveterinary medicine studies in Ethiopia documented several medicinal plants used for deworming livestock and human [10, 11, 12, 13, 14]. In spite of these valuable documentations, very few efforts have been made to scientifically evaluate these plants for their claimed activities. Most of a few works in these regard are concentrated on medicinal plants against cestodes and little is done on nematodes [15, 16]. Biffa et al [17] evaluated anthelmintic activities of three herbal preparations namely fresh leaves of *Dodonea viscosa*, *Albizia gummifera* and *Vernonia amygdalina* against mixed natural infections in sheep. They reported that *Dodonea viscosa* and *Vernonia amygdalina* have no significant anthelmintic effect while sheep treated with *A. gummifera* exhibited significant faecal egg reduction and increase in weight gain. In the current study, we have tried to screen the *in vitro* activities of seeds of four claimed medicinal plants on nematode parasite *H. contortus*.

*Croton macrostachyus*, Del (1848) (Euphorbaceae) is a shrub or tree ranging from 2-25m in height and is widely distributed in most parts of Ethiopia [18]. In Ethiopia, the fruit and decoction of the roots are used for treatment of venereal diseases, seeds for induction of abortion and leaves for treatment of constipation. The pulverized bark mixed with *Hagenia abyssinica* is used as purgative and vermifuge in different parts of Africa [19]. The leaves are reported to be used traditionally as remedy for constipation and for treatment of internal parasites of cattle in Tanzania [20]. In Ethiopia, the bark of *C. macrostachyus* is used for treatment of tapeworm infection, syphilis, and asthma in human [21]. The seed is used by local population of the Bonga area of South Ethiopia for treatment of tapeworm infection in human [22].

*Ekebergia capensis*, Sparrm (1779) (Meliaceae) is widely distributed in Ethiopia [23]. The bark mixed with other plants is used for treatment of anaplasmosis in Tanzania [20]. Extracts from *E. capensis* are used to facilitate labor in pregnant women and have been shown to have significant uterotonic activity. Decoctions made from chopped bark are also used as an emetic agent, for treatment of coughs and other respiratory complaints while leaves are used as purgative parasiticide in South Africa [24]. The concoction of *E. capensis* is used traditionally as anthelmintic for treatment of animals in central Ethiopia [11]. *E. capensis* together with *Olea capensis* are used to treat abdominal cramps by local population of the Bonga area of South Ethiopia [22]. Chemicals isolated from different parts of *E. capensis* include Limonoids, Squalene, triterpenoids and cumarins [25].

*Acacia nilotica*, Widex Del (1813) (Mimosoideae) is widely distributed in Ethiopia. The bark extract is used as soothing agent and for treatment of diabetes [26]. The powdered seed of *A. nilotica* macerated in fresh water is claimed to be useful against diarrhea in Mauritania. It is also used as anthelmintics in Nigeria in sheep and goats. Powdered seeds after removal of the outer layer are applied locally as antiseptic to treat wounds. All plants under this genus have tannin and often are source of hydrocyanic acid [27].

*Terminalia schimperiana*, Hochest (1894) (Combretaceae) is widely distributed from West Africa to Ethiopia [28]. *T. schimperiana* is used to treat cattle gastrointestinal helminths together with other plants in Cameroon [29]. *T. avicenniodes*, another species in the same genus has also

been reported to be effective against natural worm infections in sheep. The seed oil of the plant has been used as an ingredient in the treatment of rheumatic conditions, parasitic skin diseases, in the treatment of fever, jaundice, gonorrhoea, as a diuretic agent, as a mouth wash and laxative in certain African countries. The leaves have been used as a haemostatic agent and the bark as fish poison [20].

The aim of the current study was to investigate the *in vitro* anthelmintic activity of these medicinal plants on the eggs and adult *H. contortus* and to conduct qualitative phytochemical screening of these medicinal plants.

## Materials and methods

### Plant materials collection, preparation and phytochemical screening

The seeds of the plants were collected from their natural habitat from November, 2004 – April, 2005. Sample of the plant species collected were identified by plant taxonomist and voucher specimens of each species were deposited at the Herbarium of the Addis Ababa University, Biology Department. The garbled seeds were air dried at room temperature, ground and kept in amber colored bottle until processed. List of the plant species used in the study, parts used and areas of collection are shown in Table 1.

**Table 1:** Species, herbarium voucher No and areas of collection of the medicinal plants.

Species(Family)	Herbarium voucher No	Area of collection	Distance(Km)/direction from Addis Ababa
<i>C. macrostachyus</i> (Euphorbiaceae)	MG-02/05	Kuyera	240 South
<i>E. capensis</i> (Meliaceae)	MG-03/05	Assela	175 South
<i>A. nilotica</i> (Mimosoideae)	MG-05/05	Nazreth	125 East
<i>T. schimperiana</i> (Combretaceae)	MG-10/05	Wolkite	160 West

Extraction and phytochemical screening was conducted at the Drug Research Department of Ethiopian Health and Nutrition Research Institute (EHNRI). Aqueous extraction was performed by soaking a weighed amount of the dry powder (50 - 100g) in distilled water and shaking for three hours by electric shaker. The suspension was filtered through muslin gauze and the filtrate was kept in deep freezer for 24 hours, which was then lyophilized. The lyophilized dry powder was collected in stoppered sample vials, weighed and kept in a desiccator to avoid absorption of water until used. Hydro-alcoholic extraction was conducted by percolating 200-300g of the dried and powdered plant material using 80% methanol. It was then filtered through whatman filter paper No.1. The solvent was evaporated using a Rota vapor and the extract was kept in a stoppered sample vial at 4 °C until used. Preliminary qualitative screenings for major secondary metabolites of the medicinal plants were conducted according to Debella[30]. Plant materials were screened for the presence of polyphenols, cyanogenic glycosides, saponins, phytosteroides and withanoids, phenolic glycosides, flavonoids, tannins, alkaloids and antraquinone glycosides.

### Parasites

Adult female parasites of *H. contortus* were collected from the abomasums of infected sheep obtained from Addis Ababa Abattoir. The worms were washed and crushed to liberate eggs. The eggs were then cultured in a glass jar filled with autoclaved sheep faeces for eight days at room temperature. At the end of 8<sup>th</sup> day, infective larvae were harvested by rinsing the side of the culture jar with a drop of water. About 3000 larvae were inoculated to two worm free sheep that were kept indoor in separate house in the animal facilities of the AL-IPB throughout the study period. These sheep served as *H. contortus* egg donors for egg hatch assay trial.

### **Egg hatch assay**

Collection of eggs from previously mentioned donor sheep and Egg hatch assay (EHA) was conducted according to World Association for the Advancement of Veterinary Parasitology (WAAVP) guidelines, [31]. Aqueous and hydro-alcoholic extracts of the plants were used as the test treatment. Albendazole (99.8% pure standard reference) was used as positive control while untreated eggs in water were used as a negative control. About 200 eggs in 1.5ml of water were placed in each test tube. Aqueous and hydro-alcoholic extracts of plants at concentrations of 2.0, 1.0, 0.5, 0.25, 0.125, 0.0625 and 0.03125mg/ml in a total volume of 2ml was prepared together with water containing eggs. Albendazole was dissolved in Dimethyl sulfoxide (DMSO) and diluted at the concentrations of 0.5, 0.25, 0.125, 0.0625, 0.03125 and 0.0156µg/ml. The test tubes were then covered and kept in an incubator at 27 °C for 48 hrs. The experiment was replicated six times for each concentration. Hatched larvae (dead or alive) and unhatched eggs were then counted under dissecting microscope with 40X magnification.

### **Effect of plant extracts on adult worms**

Adult *H. contortus* were collected from the abomasums of sheep slaughtered at the Addis Ababa Abattoir. Immediately after slaughter, the abomasums were collected and transported to the laboratory. The parasites were then collected, washed and kept in phosphate buffered saline (PBS). Ten actively moving worms were placed in Petri dishes containing 8.0, 4.0, 2.0, 1.0, 0.5, and 0.25mg/ml of the aqueous and hydro-alcoholic extracts of plants in PBS and PBS alone for the control group in a total volume of 4 ml. Albendazole dissolved in DMSO and diluted in PBS at the concentrations of 0.5, 0.25, 0.125, 0.0625, and 0.03125mg/ml was used as a positive control. Three replications per each treatment concentration were employed. After 24 hrs, the plant extracts and albendazole were washed away and the parasites suspended in PBS for 30 minutes for possible recovery of the parasite motility. The number of motile (alive) and immotile (dead) worms were counted under dissecting microscope, and recorded for each concentration. Death of worms was ascertained by absence of motility for an observation period of 5-6 seconds. A mortality index was calculated as the number of dead worms divided by the total number of worms per Petri dish.

### **Statistical analysis**

Data from EHA were transformed by probit transformation against the logarithm of extract concentration. The extract concentration required to inhibit 50% (ED<sub>50</sub>) egg hatching was calculated using probit analysis. Comparison of mean percentages of egg hatch inhibition and mortality of adult parasites at different concentrations with the control was performed by one-way ANOVA.

## Results

### Extraction and screening of plant materials

Variation in yield among different plant species in both aqueous and hydro-alcoholic extracts was observed (Table 2). The lowest yield was recorded for hydro-alcoholic extract of seed of *E. capensis* (5.86%) and the highest yield was for hydro-alcoholic extract of the seed of *A. nilotica* (61.92%). Some plants showed higher yield in aqueous extraction while others in hydro-alcoholic extraction.

**Table 2:** Percentage yield of extracts from seeds of the medicinal plants using aqueous and hydro-alcoholic extraction methods.

Plant species	Extract type	% yield (w/w)
<i>C. macrostachyus</i>	Aqueous	10.32
	Hydro-alcoholic	16.81
<i>E. capensis</i>	Aqueous	17.48
	Hydro-alcoholic	5.86
<i>A. nilotica</i>	Aqueous	18.15
	Hydro-alcoholic	61.92
<i>T. schimperiana</i>	Aqueous	10.51
	Hydro-alcoholic	17.1

Most of the plants have major secondary metabolites like alkaloides, flavonoids, phytosteroides and withanoides. Polyphenols and flavonoids were detected in all plants tested. Saponin was not detected in all the four plants tested whereas tannin, cyanogenic glycosides and antraquinone glycosides were found only in *A. nilotica* (Table 3).

**Table 3:** Major classes of secondary metabolites found in the seeds of the four medicinal plants used in the current study.

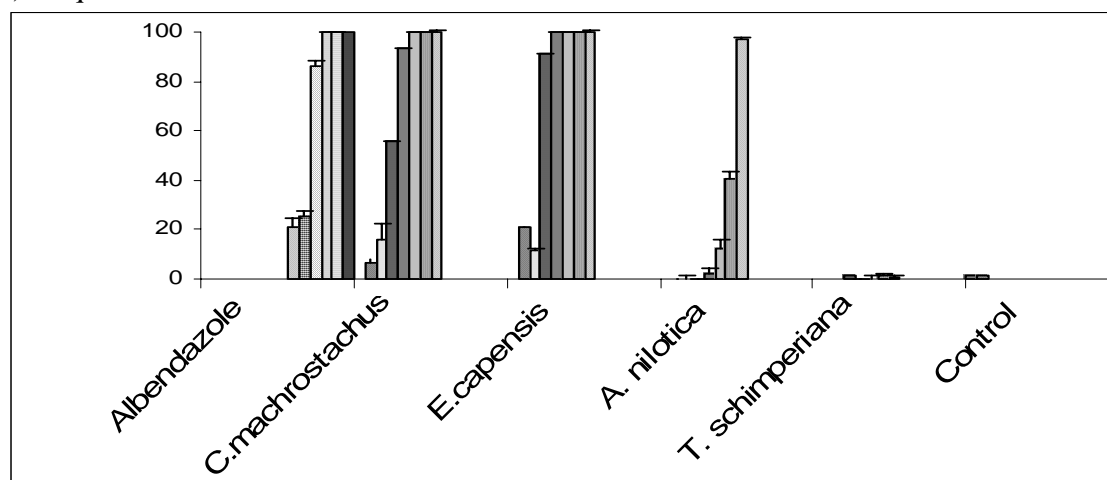
Plant species	<i>C. macrostachyus</i>	<i>E. capensis</i>	<i>A. nilotica</i>	<i>T. schimperiana</i>
Polyphenols	+	+	+	+
Cyanogenic glycosides	-	-	+	-
Saponins	-	-	-	-
Phytosteroides and Withanoides	+	+	-	+
Phenolic glycosides	-	-	+	+
Flavonoids	+	+	+	+
Tannins	-	-	+	-
Alkaloides	+	+	-	+
Antraquinone glycosides	-	-	+	-

Note + = present - = absent

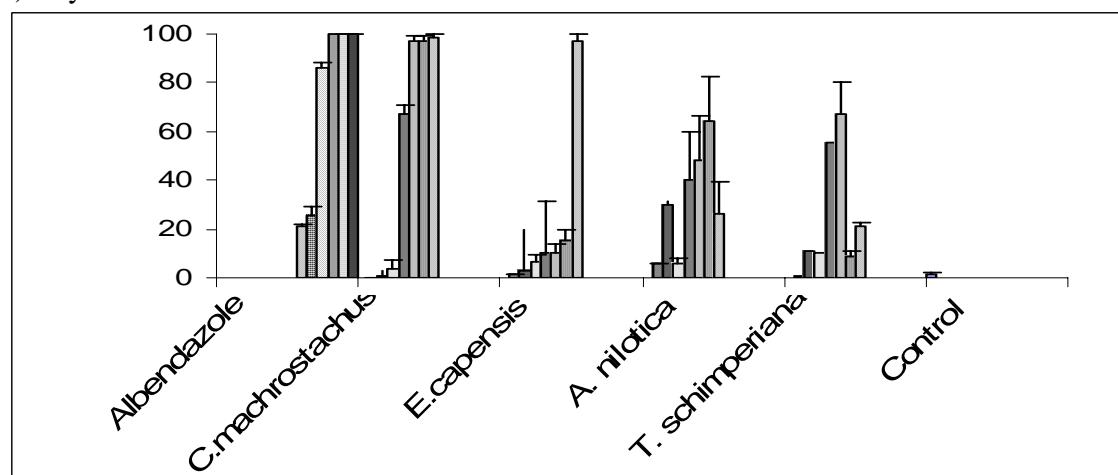
### Egg hatch assay

Both aqueous and hydro-alcoholic extracts of *E. capensis* and *C. macrostachyus* induced statistically significant egg hatching inhibition ( $p < 0.05$ ). Aqueous extract of *E. capensis* required maximum of 0.25mg/ml whereas aqueous extract of *C. macrostachyus*, required maximum concentration of 0.5mg/ml to induce 100% egg hatch inhibition. On the other hand, the hydro-alcoholic extracts of both plants did not induce complete inhibition at highest concentration tested (2mg/ml). The aqueous extract of *A. nilotica* caused dose dependent and significant ( $p < 0.05$ ) egg hatching inhibition at higher concentrations while hydro-alcoholic extract caused low activity which was not dose dependent. Both extracts of *T. schimperiana* caused low activity and the inhibition was not dose dependent (Fig. 1).

#### a) Aqueous extract



#### b) Hydro-alcoholic extract



**Figure 1:** Mean $\pm$ SEM percentage inhibition of egg hatching after 48 hours exposure of eggs of *H. contortus* to six increasing concentrations of Albendazole(0.0156,0.03125, 0.0625, 0.125, 0.25, and 0.5  $\mu$ g/ml,) and seven increasing concentrations of plant extracts (0.03125, 0.0625, 0.125, 0.25, 0.5, 1, and 2mg/ml), a) Aqueous extract ; b) Hydro-alcoholic extract.

Despite low activity in inhibition of egg hatching, aqueous extract of *A. nilotica* and hydro-alcoholic extract of *T. schimperiana* affected the survival of the hatched larvae at concentrations above 0.5mg/ml and 1mg/ml respectively.

The effective doses required to induce 50% inhibition (ED<sub>50</sub>) of egg hatching, calculated by probit analysis are shown in Table 4. Aqueous extracts of *C. macrostachyus* and *E. capensis* induced 50% inhibition at lower concentration compared to the hydro-alcoholic extract of the same plants, while hydro-alcoholic extracts of *A. nilotica* and *T. schimperiana* induced 50% inhibition at lower concentration compared to their aqueous counterparts. There was statistically significant difference between the two extract types of *C. macrostachyus*, *E. capensis* and *T. schimperiana* in ED<sub>50</sub> (p<0.05), while significant difference was not observed for *A. nilotica* (p>0.05). Based on ED<sub>50</sub>, the three most potent extracts were aqueous extract of *E. capensis* (0.06mg/ml), aqueous extracts of *C. macrostachyus* (0.1mg/ml), and hydro-alcoholic extract of *C. macrostachyus* (0.32mg/ml) in decreasing order of potency (Table 4).

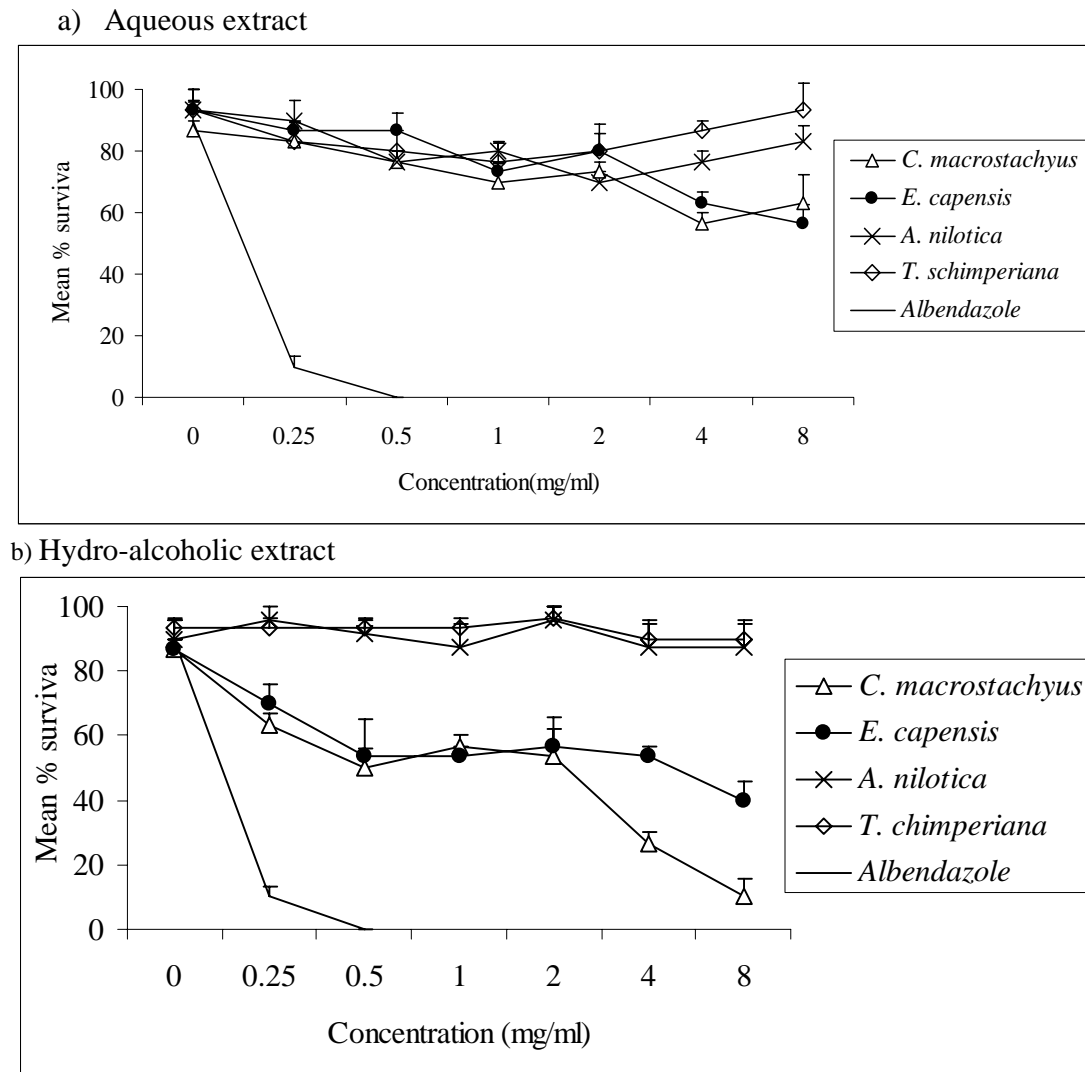
Table 4: *In vitro* anthelmintic activity of plant extracts and albendazole expressed in ED<sub>50</sub> on the eggs of *H. contortus* exposed for 48 hrs.

Plant type	Extract	ED <sub>50</sub> (LCL-UCL)
Albendazole (µg/ml)		0.04 (0.026-.051)
<i>C. macrostachyus</i> (mg/ml)	aqueous	0.10 (0.08-0.12)
	Hydro-alcoholic	0.32 (0.15-0.34)
<i>E. capensis</i> (mg/ml)	aqueous	0.06 (0.05-0.07)
	Hydro-alcoholic	1.03 (0.52-1.33)
<i>A. nilotica</i> (mg/ml)	aqueous	0.87 (0.81-1.15)
	Hydro-alcoholic	0.74 (0.22-785.04)
<i>T. schimperiana</i> (mg/ml)	aqueous	233.19(201-379 .06)
	Hydro-alcoholic	39.71(28.54-76.34)

LCL- 95% lower confidence limit, UCL-95 % upper confidence limit

### ***In vitro* effects on adult parasites**

After 24 hours of exposure of adult *H. contortus* to different concentration of plant extracts, significant and dose dependent reduction in motility /mortality was observed only for Hydro-alcoholic extracts of *C. macrostachyus* and *E. capensis*(P<0.05). Hydro-alcoholic extracts of *C. macrostachyus* and *E. capensis* produced mortality of adult *H. contortus* significantly to the level of 90 and 60 % at concentration of 8mg/ml while aqueous extract of these plants produced only 36.67 and 43.33 %, respectively at the same concentration. Both aqueous and hydro-alcoholic extracts of *A. nilotica* and *T. schimperiana* produced few mortality cases which was not statistically significant (p>0.05) compared to death of worms recorded in the control group. Albendazole, on the other hand killed the parasites in a dose dependent manner and all the worms were dead at a concentration of 0.5mg/ml with in 24 hours (Fig. 2).



**Figure 2:** Mean  $\pm$  SEM percentage survival of adult *H. contortus* after 24 hours of exposure to different plant extracts and albendazole: a) aqueous and b) hydro-alcoholic extracts

## Discussion

The variation in the yield of the aqueous and hydro-alcoholic extracts of the medicinal plants could be due to difference in the chemical composition of each plant. Some plants may contain chemicals that are more soluble in methanol while others contain chemicals that are more soluble in water. The highest extraction efficiency was that of hydro-alcoholic extract of *A. nilotica*, while the aqueous extract was three times lower. The reason for this variation could be due to high concentration of less polar organic compounds in the seeds of *A. nilotica*, which are capable of dissolving in



relatively less polar solvent methanol than water [32, 33].

Qualitative Phytochemical screening of the medicinal plants revealed that plants like *C. macrostachyus* and *E. capensis* that showed good anthelmintic activity have secondary metabolites like alkaloids and flavonoids. These classes of plant secondary metabolites are considered the sources of chemicals responsible for wide therapeutic activities of several medicinal plants [30]. The active principles that induced the observed anthelmintic activity might be found in these classes of chemicals. The presence of tannin in *A. nilotica* might also be responsible for the observed anthelmintic activity. Previous studies revealed that plants with higher content of condensed tannin have shown anthelmintic effect on different gastrointestinal nematode parasites of sheep [34, 35].

The variation in activity of the extract type of the plants might be due to difference in the proportion of the active components responsible for the tested anthelmintic activity resulting from the difference in solubility either in water or methanol. The activity of botanical compounds found from plant materials depends on the type of extractant and the method of extraction [36]. Both extracts of *C. macrostachyus*, *E. capensis* and aqueous extract of *A. nilotica* in the current study inhibited egg hatching at low concentration compared to some plants studied previously [6,37]. Increasing the concentration of the plant extracts resulted in increased inhibition of egg hatching indicating dose dependent activity. Death of the first stage larvae hatched from eggs treated with higher concentration of aqueous extract of *A. nilotica* and *T. schimperiana* indicated the larvicidal property of these extracts.

Anthelmintic drugs can reach target site in nematode parasites either by oral ingestion or by uptake/diffusion through the cuticle. However studies have shown that transcuticular diffusion is a common means of entry for non-nutrient and non-electrolyte substances in nematodes. It has also been shown that this route is predominant for the uptake of major broad-spectrum anthelmintics by different nematodes, cestode and trematode parasites as opposed to oral ingestion. Lipophilic anthelmintics such as albendazole have a greater capability to cross the external surface of the helminths than the hydrophilic compounds [38]. The possible explanation for the better activity of the hydro-alcoholic extracts of *C. macrostachyus* and *E. capensis* on adult parasites compared to the aqueous extracts could be due to easy transcuticular absorption of the hydro-alcoholic extracts into the body of the parasite than the aqueous extracts. Although distinct chemical profiles of the plant extracts are not known, in general, hydro-alcoholic extracts of plants may contain some non-polar organic chemicals with wide range of polarity than the aqueous extracts [30], rendering them higher lipid solubility than the aqueous counterparts and hence better *in vitro* anthelmintic activity.

In contrast, the aqueous extracts of the above mentioned plants have shown better activity in egg hatch assay than the hydro-alcoholic extracts. The possible explanation could be variation in the composition of active components responsible for egg hatch assay and adult parasite effect in the two types of extracts. The other reason could be due to difference in structure of the eggshell and cuticle of *H. contortus* through which absorption of chemicals take place. From these findings, it is evident that extract types of plants effective against one developmental stage of the parasite may not be effective against other developmental stage.

Both extract types of *A. nilotica* and *T. schimperiana* have shown minor effect on survival of the adult parasite at the concentrations tested, and the few deaths recorded were not in a dose dependent manner. Despite these findings, these plants have been considered principal remedies against

gastrointestinal helminths by traditional healers [27, 29]. The reason for the lack of efficacy in the current study might be due to difference in localities and age of the plant, method of preparation of the extract, species of parasite tested and/or the total absence of the real efficacy against nematodes. Worms treated by traditional healers are mainly those that are macroscopically visible after application of plant remedies. Traditional healers usually consider destrobated segments of tape worm shade post application of plant remedies as expelled worms, although this could occur irrespective of treatment [39]. Plant materials evaluated in the current study had been identified from various sources to serve as anthelmintic agents by traditional healers or farmers in different parts of Africa. Literature survey indicated no properly designed previous anthelmintic evaluation test conducted for the medicinal plants tested in the current study except putative reports of the traditional use of the plants for deworming of humans and animals.

The findings from the current study revealed that extracts from *C. macrostachyus* and *E. capensis* have shown promising *in vitro* anthelmintic activity against eggs and adult *H. contortus* which supports the traditional use of these plants as anthelmintics. Extracts from *A. nilotica* and *T. schimperiana* have shown poor activity on survival of adult parasites at concentrations tested; while aqueous extracts of *A. nilotica* have shown good activity on egg hatch inhibition. The *in vitro* methods provide a means to screen rapidly for potential anthelmintic activities of different plant extracts. However due to considerable variation in conditions encountered *in vivo* like; metabolic biotransformation, interaction with feed materials and absorption, results obtained by *in vitro* study could not be extrapolated for *in vivo* activity. The results should be ascertained by *in vivo* evaluation conducted on different animal models and different helminth parasites. *In vitro* evaluation of different plant parts and extracts that has demonstrated promising activities should also be conducted to reach at the most active part.

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