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RESEARCH ARTICLE

In Vitro and *In Vivo* Anthelmintic Activity of *Acacia nilotica* (L.) Willd. Ex Delile Bark and Leaves

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ABSTRACT

This study was carried out to assess the anthelmintic activity of *Acacia nilotica* bark and leave extracts in different solvents. Adult motility assay, egg hatch test and fecal egg count reduction test were carried out to evaluate the anthelmintic activity. Effect of plant extracts both of leaves and bark of *A. nilotica* was dose-dependent. Highest mortality of worms was observed 12 hours post-exposure @ 25 mg/ml. Extracts of leaves were more potent than the bark extracts. Ethyle acetate fractions both of bark and leaves exhibited higher anthelmintic effects compared with chloroform, petroleum spirit and aqueous fractions. Crude aqueous methanol extract (CAME) of bark (LC_{50}= 201.0032 µg/ml) had higher inhibitory effects compared with that of leaves (LC_{50}= 769.2485 µg/ml) on egg hatching. Likewise, chloroform and ethyle acetate fractions of *A. nilotica* bark exhibited higher ovicidal activity. *In vivo*, maximum reduction (72.01%) in fecal egg counts was recorded for CAME of bark followed by CAME of leaves (63.44%) @ 8 g/kg at day 12 post-treatment. Results suggest lipophilic nature of the active principles having anthelmintic efficacy in *A. nilotica* bark and leaves.

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INTRODUCTION

Nematode infections of gastrointestinal tract adversely affect productivity of small ruminants all over the world especially in tropical and sub-tropical countries. Options of using synthetic anthelmintcs are decreasing due to development of resistance in gastrointestinal nematodes of small ruminants against several families of drenches (Waller, 1994; Saddiqui *et al.,* 2010). This global problem has created interest in researches on alternates to the use of synthetic chemicals for the control of nematodes (Waller, 1999). In this regard, traditionally used ethnobotanicals with anthelmintic properties are considered among the novel approaches particularly in temperate and tropical countries (Akhtar *et al.*, 2000; Waller *et al.*, 2001). Majority of the ethnoveterinary medicine surveys and validation studies indicate much wider and effective use of plants as anthelmintics compared with other diseases/ conditions (Jabbar *et al.*, 2007; Hussain *et al.*, 2008; Al-Shaibani *et al.,* 2009; Deeba *et al.,* 2009; Sindhu *et al.,* 2010).

Leaves and legumes of Acacia species are used by the farmers for feeding small ruminants throughout the developing world. In Pakistan, most of the rangelands for grazing small ruminants are densely populated with different species of Acacia. Methanol extracts of fruit (pods with seeds) of *Acacia (A.) nilotica* (L.) Willd.ex Delile, locally known as "*Desi Kikar*", has been reported for its anthelmintic (Bachaya *et al.,* 2009) properties. Interest in *A. nilotica* has further increased due to its tannin content having been proven for anthelmintic properties (Athanasiadou *et al.,* 2000; Iqbal *et al.,* 2007). Anthelmintic activity of plants is naturally attributed to their chemical content, which may vary qualitatively and quantitatively in different parts of the same plant in the same region. These differences may be due to the type of solvent used for extraction, origin of the plant material, stage of plant development at harvesting, drying process and storage technique (Croom, 1983). This paper describes anthelmintic activity of extracts of leaves and bark of *A. nilotica* in different solvents.

MATERIALS AND METHODS

Plant material preparation

A. nilotica bark and leaves were collected directly from the plants naturally grown in farmer's fields. Voucher specimens (# bark 87a/2008 and leaves 87b/2008) were kept at the Herbarium, Ethno veterinary Research and

Development Center, Department of Parasitology, University of Agriculture, Faisalabad (UAF) (Pakistan) after authentication of plants from a botanist at the Department of Botany, UAF. The plant materials were dried under shade and ground into fine powder. Crude aqueous-methanol extracts (CAME) were prepared following the methods of Tabassam *et al.* (2008). Fractionation of CAME was done using three different organic solvents, i.e., chloroform, petroleum spirit and ethyle acetate (Williamson *et al.*, 1998). Rotary evaporator was used for evaporation of solvents under reduced pressure at 35°C and stored at 4°C until used.

Anthelmintic activity

Anthelmintic activity of the extracts of plants was assessed *in vitro* using adult motility assay and egg hatch test, and *in vivo* using fecal egg count reduction test.

Parasites

Adult *Haemonchus (H.) contortus* worms were obtained from the abomasal contents of slaughtered sheep. Some of the worms were kept separate to be used in adult motility assay; whereas, from the remaining worms, females were separated and crushed in mortar and pestle to liberate the eggs, which were cultivated *in vitro* for infective larvae. Two lambs, naive to *H. contortus*, were infected with these larvae. Fecal samples were collected and cultured again on day 25 post-infection to harvest infective larvae of *H. contortus* (Rossanigo and Gruner, 1995). These larvae were then used to infect two new naive lambs. Fecal samples from these two lambs called as "donor lambs" were used to obtain eggs for egg hatch test.

Adult motility assay

Mature live *H. contortus* from sheep were used to determine the effect of plant extracts by the method described previously by Singh *et al.* (1985). For this purpose, abomasums were collected from sheep freshly slaughtered in the local abattoir and incised for recovering the immature worms. The worms were washed and finally suspended in phosphate buffered saline (PBS). Ten worms were exposed in three replicates to each of the following treatments in separate Petri dishes/test tubes at room temperature (25-30°C): crude aqueous methanol extract, petroleum spirit fraction, chloroform fraction and ethyl acetate fraction each @ 50, 25, 12.5, 6.25, 3.12 and 1.56 mg/ml; Levamisol @ 0.55 mg/ml and PBS.

The inhibition of motility and/or mortality of worms kept in different treatments were used as criterion for the anthelmintic activity. The motility was observed on 0, 2, 4, 6, 8, 10 and 12 hr intervals. Finally, the treated worms were kept for five minutes in the lukewarm fresh PBS for the revival of motility. The number of dead and survived worms was recorded for each treatment.

Egg hatch test (EHT)

Haemonchus contortus eggs were isolated from feces of donor lambs following Hubert and Kerbouef (1992) and EHT was performed in triplicate as described by Coles *et al.* (1992). Briefly, stock solutions 12000 µg/ml of all the extracts (crude aqueous methanol, chloroform, petroleum spirit, ethyle acetate) were prepared in 0.1-0.5% DMSO depending on the solubility of plant extracts. Subsequently, stock solution was diluted serially (12000–1.2 µg/ml) in the same diluent. Similarly, stock solution of 25 µg/ml of oxfendazole was prepared in 0.1% DMSO and diluted serially (25.0–0.0025 µg/ml). Approximately, 250 freshly collected eggs of *H. contortus* were distributed in each well of a 24-flat-bottomed microtitration plate (Flow Laboratories) and exposed to different concentrations of extracts and oxfendazole described above. The negative control well received 0.1% DMSO (diluent of extracts/fractions and oxfendazole) only. The microtitration plates were incubated at 28°C for 48 h to for hatching of the eggs. After 48 h, a drop of Lugol's iodine solution was added to each well of the microtitration plate. All the eggs and first-stage larvae (L1) in each plate were counted to assess the effect of different treatments on the hatching of eggs.

Fecal egg count reduction test

Animals

The *in vivo* trial was conducted at a private small ruminants farm in Roshan Wala in the vicinity of Faisalabad (Pakistan). Eighty-four male sheep (young stock≤1 year), weighing 20-24 kg, naturally parasitized with gastrointestinal nematodes (GINs) were selected. The experimental animals were vaccinated against enterotoxemia and pleuropneumonia vaccines supplied by the Veterinary Research Institute, Lahore (Punjab, Pakistan). Nematode infection and eggs per gram of feces were confirmed before the beginning of study following the standard parasitological procedures of fecal examination (Urquhart *et al.*, 2003). Coproculture was carried out to ascertain the nematode species composition and identification of larvae using standard description of MAFF (1986). Animals were found to have mixed infection of GINs including *Teladorsagia circumcincta*, *H. contortus*, *Trichostronglyus* spp., and *Trichuris ovis*. The experimental animals were penned singly by treatment and no physical contact was possible between the animals from different treatment groups. Sheep were kept on plastered floor and fed grass and water *ad libitum*.

Experimental design

Experimental sheep were randomly divided into 14 groups of six animals each using completely randomized design and assigned to different treatments *per os* as a single dose as follows:

Groups	Treatments
1	Untreated control
2	Levamisole HCl (Nilverm® 1.5%, w/v; ICI Pakistan Limited, Animal Health Division) at 7.5 mg/kg body weight (b.wt.)
3	*A. nilotica* leaves crude powder (CP) @ 1 g/kg b.wt.
4	*A. nilotica* leaves CP @ 4 g/kg b.wt.
5	*A. nilotica* leaves CP @ 8 g/kg b.wt.
6	*A. nilotica* leaves crude aqueous methanolic extract (CAME) @ 1 g/kg b.wt.
7	*A. nilotica* leaves CAME @ 4 g/kg b.wt.
8	*A. nilotica* leaves CAME @ 8 g/kg b.wt.
9	*A. nilotica* bark crude powder (CP) @ 1 g/kg b.wt.
10	*A. nilotica* bark CP @ 4 g/kg b.wt.
11	*A. nilotica* bark CP @ 8 g/kg b.wt.
12	*A. nilotica* bark crude aqueous methanolic extract (CAME) @ 1 g/kg b.wt.
13	*A. nilotica* bark CAME @ 4 g/kg b.wt.
14	*A. nilotica* bark CAME @ 8 g/kg b.wt.

Dose of different treatments for animals was calculated according to their bodyweight and administered *per os* to the individual animals. Fecal sample of each experimental animal was collected in the morning, starting from day 0 pre-treatment and at days 4, 8 and 12 post-treatment (PT). Eggs per grams of feces (EPGs) were determined by the McMaster Egg Counting Technique (Urquhart *et al*., 2003). Egg count percent reduction (ECR) was calculated by the following formula:

$$ECR\ (\%) = \frac{\text{Pre - treatment egg count per gram - post - treatment egg count per gram}}{\text{Pre - treatment egg count per gram}} \times 100$$

Statistical analyses

Data from egg hatch test were transformed by probit transformation against the logarithm of plant extract (Hubert and Kerboeuf, 1992). Probit transformation was performed to transform a typical sigmoid curve dose response to a linear function. The lethal concentration 50 (LC_{50}) of extract concentration required to prevent 50% hatching of eggs (in case of egg hatch test) was calculated from the linear regression (for y = 0 on the probit scale). In adult motility assay, comparison between means of dead worms was made using DMR Test. Results of fecal egg count reduction test were expressed as eggs per gram (Mean±SE) of feces and means were compared by using DMR Test (SAS, 1998).

RESULTS

Adult motility assay

Effect of all plant extracts both of leaves and bark of *A. nilotica* was dose-dependent. Highest mortality ($P<0.05$) of worms was observed 12 hours post-exposure @ 25 mg/ml (Table 1). Extracts of leaves were more potent than the bark extracts. Ethyle acetate fractions both of bark and leaves exhibited higher anthelmintic effect compared with chloroform, petroleum spirit and aqueous fractions. There was 100% mortality of worms in Levamisole (used as a reference drug) within 2 hours post-exposure. There was no mortality of worms kept in PBS till 12 hours post-experiment.

Egg hatch test

Inhibitory effect of different extracts of *A. nilotica* on percent egg hatching was very low as compared to oxfendazole. CAME of bark (LC_{50}= 201.0032 µg/ml) was found to has higher inhibitory effects compared with that of leaves (LC_{50}= 769.2485 µg/ml) on egg hatching. Chloroform and ethyle acetate fractions of *A. nilotica* bark exhibited good ovicidal activity. The data of correlation of regression revealed a dose dependent response of extracts both for bark and leaves. Lethal concentration 50 (LC_{50}) for the inhibition of egg hatching are shown in Table 2.

Fecal egg count reduction test

Both crude powder and crude methanol extracts of *A. nilotica* bark and leaves exhibited a dose dependent anthelmintic activity (Fig. 1). The maximum reduction (72.01%) in fecal egg counts was recorded for CAME of bark followed by CAME of leaves (63.44%) @ 8 g/kg at day 12 post-treatment.

DISCUSSION

All the treatments based on extracts/fractions/CP of *A. nilotica* bark and leaves exhibited anthelmintic activity. Leaves were found to have higher effects *in vitro* against adult worms; whereas, bark proved to be a better ovicidal in EHT. Ethyle acetate fractions of both bark and leaves demonstrated higher efficacy than those of other fractions tested in this study. *In vivo*, *A. nilotica* bark was more effective in reducing the eggs per gram of feces compared with *A. nilotica* leaves. Moreover, CAME of both bark and leaves were more effective compared with CP.

Fruits (pods with seeds) of *A. nilotica* have been reported for their anthelmintic (Bachaya *et al.,* 2009) activity. Major phytochemicals in Acacia spp. are flavonoid and tannins (Tindale and Roux, 1969; Malan and Roux,

Table 1: *In vitro* effect of different fractions of crude aqueous methanol extracts of *Acacia nilotica* bark and leaves on survival of *Haemonchus contortus* of sheep

Treatments[1]	Number of dead worms (Mean±SE) at different hours					
	Acacia nilotica bark			*Acacia nilotica* leaves		
Hours post-exposure	0 hr	6 hr	12 hr	0 hr	6 hr	12 hr
Chloroform						
1.56 mg/ml	0.00±0.00^{k}	0.00±0.00^{k}	1.00±0.000^{hk}	0.00±0.00^{i}	0.00±0.00^{i}	0.67±0.333^{hij}
6.25 mg/ml	0.00±0.00^{k}	0.67±0.333^{ijk}	1.67±0.333^{ghi}	0.00±0.00^{i}	1.00±0.000^{gi}	2.00±0.000^{efg}
25 mg/ml	0.00±0.00^{k}	2.00±0.000^{fgh}	4.00±0.577^{de}	0.00±0.00^{i}	2.33±0.333^{ef}	4.00±0.577^{c}
Ethyle acetate						
1.56 mg/ml	0.00±0.00^{n}	0.33±0.333^{mn}	1.00±0.000^{kn}	0.00±0.00^{m}	0.67±0.333^{klm}	1.67±0.333^{ijk}
6.25 mg/ml	0.00±0.00^{n}	1.00±0.000^{kn}	2.33±0.667^{g-j}	0.00±0.00^{m}	1.67±0.333^{ijk}	3.33±0.333^{fg}
25 mg/ml	0.00±0.00^{n}	2.67±0.333^{ghi}	5.00±0.577^{de}	0.00±0.00^{m}	4.00±0.577^{ef}	6.67±0.333^{c}
Petroleum spirit						
1.56 mg/ml	0.00±0.00^{h}	0.00±0.00^{h}	0.33±0.333^{gh}	0.00±0.00^{g}	0.00±0.00^{g}	0.00±0.000^{g}
6.25 mg/ml	0.00±0.00^{h}	0.33±0.333^{gh}	1.00±0.000^{efg}	0.00±0.00^{g}	0.33±0.333^{fg}	1.33±0.333^{ef}
25 mg/ml	0.00±0.00^{h}	1.00±0.000^{efg}	1.67±0.333^{de}	0.00±0.00^{g}	2.33±0.333^{d}	3.00±0.577^{d}
Aqueous						
1.56 mg/ml	0.00±0.00^{i}	0.00±0.00^{i}	0.00±0.00^{i}	0.00±0.00^{i}	0.00±0.00^{i}	0.67±0.333 ^{ghi}
6.25 mg/ml	0.00±0.00^{i}	0.00±0.00^{i}	1.00±0.000^{gh}	0.00±0.00i	0.67±0.667^{ghi}	1.33±0.333 $^{e-h}$
25 mg/ml	0.00±0.00^{i}	1.33±0.333^{fg}	2.33±0.333^{cd}	0.00±0.00i	1.67±0.333^{efg}	2.33±0.667 ^{de}

[1]Results of adult motility assay at 50, 12.5 and 3.12 mg/ml, and 2, 4, 8 and 10 hours post-exposure have not been shown as trend of anthelmintic effects was similar to that given in the table for other hours/concentrations.

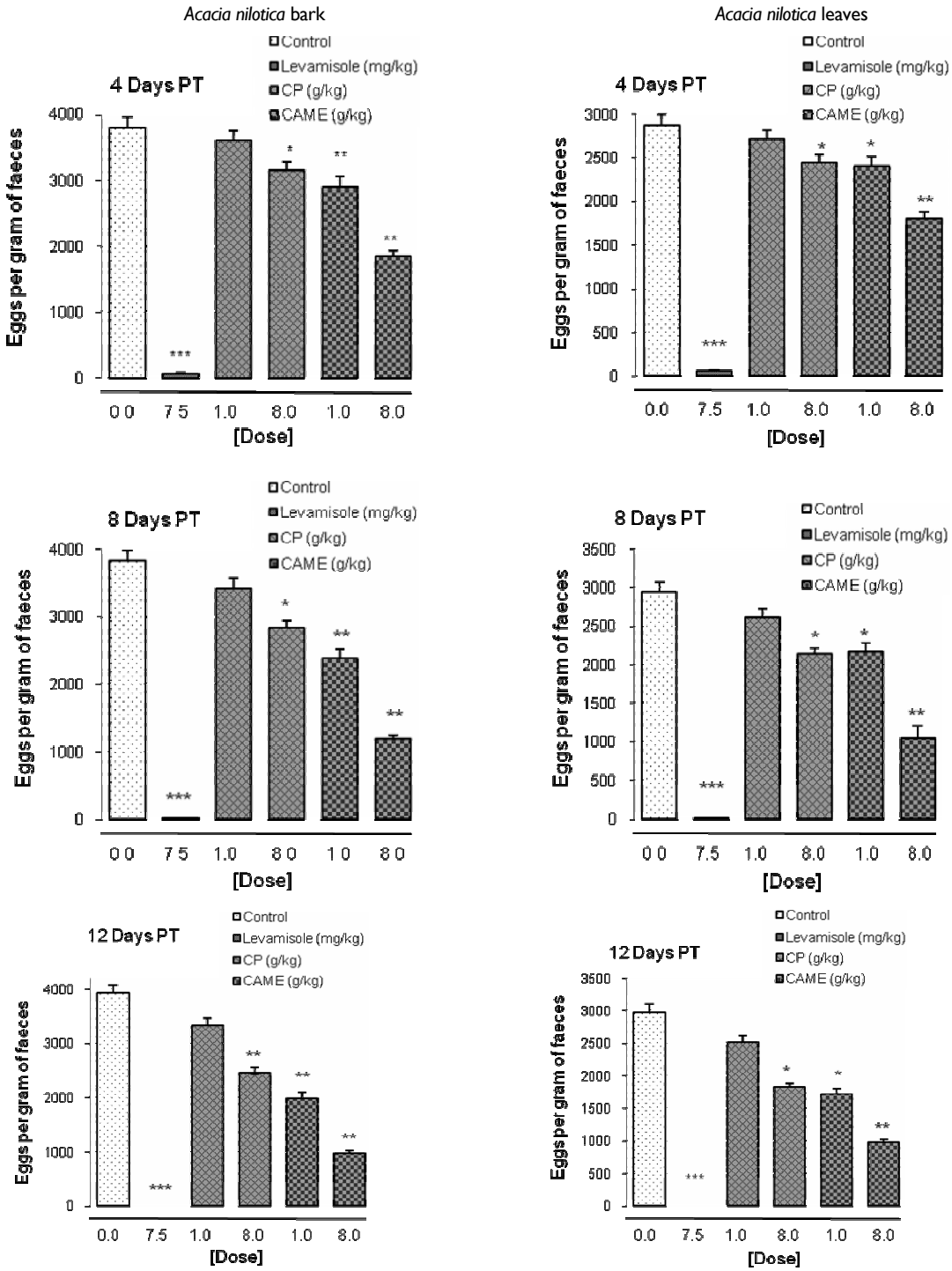

Fig. 1: Bar diagrams showing the dose-dependent (1.0–8.0 g/kg) anthelmintic activity of *Acacia nilotica* bark and leaves as crude powder (CP) and crude aqueous-methanol extract (CAME) in sheep naturally infected with mixed species of gastrointestinal nematodes at 4, 8 and 12 days post-treatment (PT). Activity of CP and CAME is compared with that of positive control levamisole (7.5 mg/kg). Values shown are mean±S.E., n=6; *P < 0.05 and ** P < 0.005, vs. negative control

1975; Devi and Prasad, 1991), cyanogenic glucosides (Secor *et al.*, 1976), free amino acids (Evans and Bell, 1979), acacipetalin (Seigler *et al.*, 1978), labdane diterpenes (Forster *et al.*, 1985), and proanthocyanidins and other phenolics (Dube, 1993). The anthelmintic efficacy of *A. nilotica* may be attributed to an individual or a combined effect of the compounds or chemical groups given above. The antimicrobial activity of flavones, flavonoids, and flavonols is probably due to their ability to complex with extracellular and soluble proteins and to complex with bacterial cell walls, more lipophilic flavonoids may also disrupt microbial membranes (Tsuchiya *et al.*, 1996). The mechanism of action of the antimicrobial activity of terpenoids and essential oils (Suresh *et al.*, 1997; Amaral *et al.*, 1998) is not fully understood but is speculated to involve membrane disruption by the lipophilic compounds.

Table 2: Effect of different extracts of *Acacia nilotica* on hatching of *Haemonchus contortus* eggs

Plant extracts	LC_{50} µg/ml	Regression values and correlation of regression
Crude aqueous methanol extract		
Acacia nilotica bark	201.0032	y = -0.4196x + 7.2252, R2 = 0.9838
Acacia nilotica leaves	769.2485	y = -0.3485x + 7.0513, R2 = 0.9358
Fractions		
Acacia nilotica leaves		
Petroleum spirit fraction	8408.9702	y = -0.2603x + 6.8025, R2 = 0.9907
Aqueous fraction	1046.5714	y = -0.3804x + 7.2899,R2 = 0.9810
Chloroform fraction	156.5536	y = -0.4295x + 7.2311, R2 = 0.9902
Ethyle acetate fraction	129.4961	y = -0.4414x + 7.2566, R2 = 0.9909
Acacia nilotica bark		
Petroleum spirit fraction	289.2595	y= -0.3957x + 7.161, R2 = 0.9935
Aqueous fraction	222.8358	y = -0.4427x + 7.3676, R2 = 0.9812
Ethyle acetate fraction	52.5873	y = -0.6302x + 7.9751, R2 = 0.9636
Chloroform fraction	50.9734	y = -0.4346x + 7.0458, R2 = 0.9332
Oxfendazole	0.114	$y = -0.7712x + 5.4081$, R2 = 0.8871

Tannins have been reported to complex with polysaccharide (Ya *et al.*, 1988). Condensed tannins have been determined to bind cell walls of ruminal bacteria, preventing growth and protease activity (Jones *et al.*, 1994). At least two studies have shown tannins to be inhibitory to viral reverse transcriptases (Kaul *et al.*, 1985; Nonaka *et al.*, 1990). One of the molecular actions of tannins is to complex with proteins through so-called nonspecific forces such as hydrogen bonding and hydrophobic effects, as well as by covalent bond formation (Haslam, 1996; Stern *et al.*, 1996). There are numerous reports indicating direct or indirect anthelmintic effects of condensed tannins (CT) (Aerts *et al.*, 1999; Kahn and Diaz-Hernandez, 2000; Athanasiadou *et al.*, 2001; Niezen *et al.*, 2002).

Variation in the anthelmintic activity of the extracts/fractions tested in this study may be attributed to the disparity in the targets on the stage of parasites for action of the compounds, qualitative and/or quantitative differences in the active principles present in bark and leaves, and their solubility in different solvents. Based on the mode of action, the currently in market anthelmintics can be divided into nicotinic agonists, acetylcholinesterase inhibitors, GABA agonists, GluCI potentiators, calcium permeability increasers, β-tubulin binders, proton ionophores, inhibitors of malate metabolism, inhibitors of phosphoglycerate kinase and mutase, inhibitors of arachidonic acid metabolism and stimulators of innate immunity. Therefore, different compounds/active principles of plant extracts/fractions may have different targets to exert anthelmintic effect on eggs, larvae and adults. The known target sites on parasites are solely proteins and include ion channels, enzymes, structural proteins, transport molecules, etc. (Lacey, 1988; Geary *et al.*, 1992; Martin, 1993; Kohler, 2001).

The targets to exert anthelmintic effects may differ in various parasite stages. Most of the screening *in vitro* tests are most easily applied to the free-living stages of parasite species, i.e., eggs, larvae, etc.The ultimate use of the anthelmintic, however, will be directed at the parasitic stages (Grady and Kotze, 2004). The neurotoxic effects of drug may be similar in free-living and parasitic stages; whereas, biochemistry and physiology of free-living and parasitic stages differ in many aspects relevant to potential anthelmintic targets or potential detoxification mechanisms. For example, changes in energy metabolism from aerobic to anaerobic during the transition from free-living to parasitic life stages (Komuniecki and Komuniecki, 1995), decrease in oxidative detoxification capability in parasitic stages compared to free-living (Kotze, 1997).

In conclusion, in spite of differences in the biology of bacteria, fungi, protozoa, and helminths, there are some common targets among them which can also be utilized by the compounds having anthelmintic activity. These may include inhibition of enzymes, complexing with proteins, polysaccharide, formation of ion channels, etc. Such targeted interventions may result in disturbing the normal biochemical and physiological processes leading to starvation, structural changes, neuromuscular interruptions, and other effects on helminths. In fact, most of these are the known target sites for commonly used anthelmintics (Kohler, 2001; Mottier *et al.*, 2006). Nevertheless, *A. nilotica* bark and leaves extracts have demonstrated anthelmintic activity in the present study.

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