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In vitro anthelminthic activities of four medicinal plants against *Haemonchus contortus*

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In vitro **experiments were conducted to determine the anthelmintic effects of crude aqueous extracts of the leaves of *Carissa spinarum* and *Azadrichta indica*, fruits of *Phytolacca dodecandra* and stem bark of *Acacia tortilis* on eggs and adults of *Haemonchus contortus* using egg hatch assay and mortality of adult parasite. Extracts of the leaves of *C. spinarum* and *A. indica* inhibited hatching of egg at concentration less than or equal to 1 mg/ml. Low egg hatch inhibition were observed for extracts of *A. tortilis* (100%) and *P. dodecandra* (99.4%) at the maximum concentration tested (2 mg/ml). Of the plants tested, extracts of *C. spinarum* and *A. indica* showed very good activity against the adult worms of *H. contortus*, mortality raised to the levels of 96.8 and 93.9%, respectively, at concentration of 4 mg/ml. *P. dodecandra* and *A. tortilis* produced mortality of 68.1 and 53.03% of adult *H. contortus* at 4 mg/ml concentration, respectively. Albendazole killed the parasites in a dose dependant manner and all the worms were dead at a concentration of 0.5 mg/kg within 24 h. The overall findings of the current study indicated that most of the plants have potential anthelmintic effect warranting further *in vitro* and *in vivo* evaluation.**

Key words: Anthelmintic, *Haemonchus contortus*, *in vitro* experiment, plant extracts.

INTRODUCTION

Helminthosis play a crucial role in small ruminant production leading to enormous economic losses particularly in areas where extensive grazing is practiced (Tembely et al., 1994; Waller, 1997). It causes loss of production directly and indirectly. The direct loss is manifested through mortality, loss of blood and plasma protein by blood sucking behavior of the parasites and leakages into gastrointestinal tract, depression of mineral level and diarrhea, all contributing to weight loss, reduced milk and wool production (Soulsby, 1986). The indirect economic impact is manifested by increased cost of control strategies (anthelmintics, labor, drenching equipments) and other parasite-related penalties such as delay in achieving target weights, increased feed requirements to achieve target weight and reduced quality of carcass and predisposition to other diseases (Kassai, 1999). *Haemonchus contortus*, the causative agent of haemonochosis, is one of the most pathogenic and highly prevalent nematode parasites of small ruminants particularly in the tropics and subtropics. Haemonchosis is characterized by anemia attributable to blood loss via blood sucking activities of worms in the abomasums (Soulsby, 1986); causing acute disease and high morality in all classes of livestock (Allonby and Urquhart, 1975). It is one of the top 10 constraints of sheep and goat production in East Africa (Perry et al., 2002). In Kenya, haemonchosis alone was estimated to cause an annual loss of 26 million dollar in sheep and goat production (Allonby and Urquhart, 1975).

Commercial anthelmintics have been used for some

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decades throughout the world to minimize the losses caused by helminth infections (Waller, 1997). The threats of anthelmintic resistance, risk of residue, availability and high cost, especially to farmers of low income in developing countries, have led to the notion that sustainable helminth control can not be achieved with commercial anthelmintics alone. Other alternative options like biological control, vaccine and traditional medicinal plants are being examined in different corners of the world (Bain, 1999; Chandrawathani et al., 2003; Githiori, 2004; Waller and Thamsborg, 2004). Evaluation of the activities of medicinal plants claimed for anthelmintic property is getting attention these days (Gathuma et al., 2004; Githiori, 2004). Rich literature is available on ethno veterinary use of medicinal plants as anthelmintics. Traditionally in Ethiopia, the leaves, root and bark of *Carissa spinarum* are used against GIT parasites and ring worm (Sory, 1999; Hayatu, 2003). The leaves and fruits of *Phytolacca dodecandra* are used against endo-parasites (Belay, 2004), and the leaves as antiseptic (Haylessielassie, 2004). The root, bark and inner bark of *Acacia tortilis* are used to treat diarrhea (Sory, 1999; Beyecha, 2004) and the bark is used topically to treat ring worm (Hayatu, 2003). The roots, leaves and bark of *Azadrichta indica* used against endo- and ecto parasites (Sory, 1999; Beyecha, 2004). However, most of the reports did not provide detailed information on the part of the plants used and method of preparation. Often, no validation of the effect against the disease conditions is provided.

The present study was, therefore, carried out to assess the anthelmintic activities of aqueous extracts of four Ethiopian plants, *C. spinarum* L. (Apocynaceae), *A. indica* A. Juss (Meliaceae), *P. dodecandra* L'Herit (Phytolaccaceae) and *A. tortilis* (Forssk) Hayne (Fabaceae), *in vitro* using eggs and adults of live *H. contortus*. Aqueous extracts of these plants have not previously been evaluated for their activity against *H. contortus*.

MATERIALS AND METHODS

Plant collection

Selection of plants was based on literature survey on traditional uses in Ethiopia and other parts of the world. The leaves root and bark of *C. spinarum* are used against GIT parasites and ring worm (Sory, 1999; Hayatu, 2003). The leaves and fruits of *P. dodecandra* are used against endoparasites (Belay, 2004), and the leaves as antiseptic (Haylessielassie, 2004). The root, bark and inner bark of *A. tortilis* are used to treat diarrhea (Sory, 1999; Beyecha, 2004) and the bark is used topically to treat ring worm (Hayatu, 2003). The roots, leaves and bark of *A. indica* used against endo- and ectoparasites (Sory, 1999; Beyecha, 2004). For the *in vitro* test, leaves of *C. spinarum* and *A. indica*, fruits of *P. dodecandra* and stem bark of *A. tortilis* were collected from different localities of the country between November 2007 and January 2008. All the plants were identified by a plant taxonomist and voucher specimens of each species were deposited at the Aklilu Lemma Institute of Pathobiology (ALIPB), Addis Ababa University. The collected plant parts were air-dried at room temperature, ground and stored until extraction.

Extraction method

Aqueous extracts were produced at the Aklilu Lemma Institute of Pathobiology (ALIPB). Fifty grams of the dry powder of each plant sample was socked in distilled water and shaken for 24 h by electric shaker. The suspension was filtered using filter paper and the filtrate kept in deep freezer (-70°C) for 24 h, and then lyophilized using lyophilizer. The lyophilized extract (freeze-dried) dry powder was then collected, weighed and kept in a dry place to avoid absorption of water until being used for the test.

Parasites

Adult female parasites of *H. contortus* were collected from the abomasums of infected sheep obtained from Addis Ababa Abattoir's Enterprise. *H. contortus* is reported to have developed resistance to most anthelmintics used in the country according to unpublished reports of the Ethiopian Ministry of Agriculture and Rural Development. The worms were washed and crushed to liberate eggs. The eggs were then cultured in a glass jar filled with autoclaved horse faeces for eight days at room temperature. At the end of 8th day, infective larvae were harvested by rinsing the side of the culture jar with a drop of water. About 3000 larvae were then orally inoculated to worm free sheep (5 g of feaces was taken from the rectum for floatation and concentration techniques to make sure that the sheep were worm free) of ages between 4 and 6 months, kept in-door in separate house in the animal facilities of the ALIPB throughout the study period. These sheep served as *H. contortus* egg donors for subsequent *in vitro* trials.

In vitro experiments

Collection of eggs

Briefly, faecal pellets were collected from the rectum of donors' sheep and placed in small bucket. Warm water was slowly added to the faeces and the pellets stirred until a relatively uniform homogenate was obtained, liquid suspension was obtained. The suspension was filtered through sieve with 3 mm aperture. The resulting suspension was again made to pass through a sieve of 150 µm pore size. The suspension was then poured into 15 ml test tubes and centrifuged for 2 min at 377 g and the supernatant decanted. The tube was agitated by vortex mixer to loosen the sediment. Saturated sodium chloride was then added to the test tube until the meniscus forms above the test tube on which the cover slip was placed. After 3 to 5 min, the cover slip was carefully taken off the tube and eggs washed into glass centrifuge tubes filled with water and centrifuged for 2 min at 377 g. Most of the water was then decanted and the number of eggs per ml was determined before diluting it to the required concentration for use in 'egg hatch assay'.

Egg hatch assay

The 'egg hatch assay' was conducted according to World Association for the Advancement of Veterinary Parasitology (WAAVP) guidelines (Coles et al., 1992). The eggs used for this test were aged less than 3 h. Aqueous extracts of the plant materials were used as the active treatment. Albendazole (99.3% pure standard reference) obtained from the Ethiopian Drug Administration and Control Authority (DACA) was used as positive control while untreated eggs in water were used as negative control. The test was conducted in 4 ml test tubes. In the assay, approximately 100 to 120 eggs in 1.5 ml water were placed in each test tube. Each plant extract was serially diluted in a total volume of 2 ml distilled water to make concentrations of 0.0625, 0.125, 0.25, 0.5, 1 and 2 mg/ml together with water containing the eggs. Albendazole origin-

nally dissolved in dimethyl sulfoxide (DMSO) and distilled water at the concentrations of 0.0625, 0.125, 0.25, 0.5, 1 and 2 µg/ml was used. The test tubes were then covered and kept in incubator at 27°C for 48 h. The experiment was conducted in duplicates for each concentration and replicated three times. Hatched larvae (dead or alive) and unhatched eggs were then counted under dissecting microscope at 40X magnification.

In vitro effect of plant extracts on adult worms

Adult *H. contortus* were collected from abomasums of the sheep slaughtered at the Addis Ababa Abattoir's enterprise. Immediately after slaughtering, the abomasums were collected and transported to ALIPB laboratory. The collected parasites were washed and kept in phosphate buffered saline (PBS). The test was performed in 5 cm diameter plastic petridish. Nine to eleven worms were placed in petri dishes filled with 0.25, 0.5, 1, 2, 4 and 8 mg/ml of the extract of the plant material in PBS and those with PBS alone (to serve as a control group) in total volume of 4 ml. Albendazole dissolved in DMSO at the concentration of 0.0625, 0.125, 0.25, 0.5, 1 and 2 mg/ml was also used as a positive control. Each concentration was tested in triplicate. After 24 h, the extract was washed away and the parasites were re-suspended in PBS for 30 min for possible recovery of the parasite motility. Finally, the number of motile (alive) and immotile (dead) worms were counted under dissecting micro-scope and recorded for each concentration. A mortality index was calculated as the total number of dead worms divided by the total number of worms per petri dish.

Data management and statistical analysis

Comparison of mean percentages of egg hatch inhibition and adult mortality, at different concentrations with the control, was performed by one-way ANOVA. All statistical analysis was performed by SPSS version 13.0. The Post Hoc statistical significance test employed was list square difference (LSD), the difference between the means were considered significant at $p < 0.05$.

RESULTS

In vitro experiments

Egg hatch assay

All the four extracts of the plants exhibited good activities against eggs of *H. contortus*; although, there was variation in doses required for each type of extract. In all plant extracts, the maximum concentration used in the study (2 mg/ml) induced nearly 100% egg hatch inhibition. Extract of *C. spinarum* induced 100% egg hatch inhibition at a concentration of 0.5 mg/ml, the least concentration among all plant extracts tested to bring about the same effect, while *P. dodecandra* was the weakest plant that gave 99.4% inhibition at 2 mg/ml concentration. Albendazole induced 100% egg hatch inhibition at a concentration of 0.25 µg/ml (Table 1).

In vitro effects of plant extract on adult parasites

All the extracts showed inhibitory effect on the survival of *H. contortus* in a dose dependant manner. *C. spinarum*, *A. indica* and *A. tortilis* produced mortality of adult *H. contortus* significantly to the level of 96.8, 93.9 and 53.03%, respectively, at a concentration of 4 mg/ml while *P. dodecandra* produced 68.1% mortality at the same concentration. Albendazole, on the other hand, brought about 100% parasite mortality at a concentration of 0.5 mg/kg within 24 h (Table 2).

DISCUSSION

In this study, significant variation in the yield of extracts among the different medicinal plants was observed. The fruits of *P. dodecandra* gave the highest yield while the lowest was observed for the leaves of *C. spinarum*. Apart from the difference in species, plant part used, age, harvest season and habitat could also contribute to the variation in biochemical profiles and yields (Habtemariam et al., 1994). Several *in vivo* and *in vitro* techniques have been developed to detect anthelminitic resistance in nematodes (Craven et al., 1999). However, *in vivo* tests are not the best model to screen plants extracts with anthelmintic activity, since these tests are time-consuming, expensive and present low precision and reproducibility due to inter-animal variation and pharmacodynamics of the drugs in the host (Lacey et al., 1990). Although, the adult nematode is the major target for the chemotherapy, gastrointestinal nematode parasites cannot yet be raised in continuous culture (Geary et al., 1999). Thus, the lack of a culture system yielding adult of any nematode parasite prevents a preliminary study of the effect of medicinal plants on this stage. On the other hand, the *in vitro* tests using free living stages of parasitic nematodes offer a means of evaluating the anthelmintic activity of new plant compounds, as already reported by various authors (Asase et al., 2005). Most of the plant extracts in the current study inhibited 100% egg hatchability at low concentration (2 mg/ml) as compared to other plants studied previously. For example, 7.1 mg/ml of aqueous extract of *Annona senegslensis*, 2.5 mg/ml of essential oil of *Ocimum gratissimum* and 50 mg/ml methanol extract of *Spigelia anthelmia* inhibited 11.5% (Alawa et al., 2003), 96.94% (Pessoa et al., 2002) and 97.4% (Asase et al., 2005) of egg hatchability, respectively.

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 Ethiopia. The leaf, root and bark of *C. spinarum* is used against GIT parasites and ring worm (Sory, 1999; Hayatu, 2003). This finding is confirmed by the current study that the leaves of *C. spinarum* have a higher effect on the survival of the parasite showing 100% inhibition of eggs hatchability at concentration of 0.5 mg/ml and 98.8% adult mortality of *H. contortus* at concentration of 4 mg/ml. The roots, leaves and bark of *A. indica* are used traditionally against endo- and ecto-parasites (Sory, 1999; Beyecha, 2004) and the present study also showed that the aqueous extract from the leaves of this plant exhibited 100% inhibition of egg hatchability at concentration of 1 mg/ml and 93.9% of adult mortality of *H. contortus* at concentration of 4 mg/ml. The leaves and

Table 1. Mean percentage inhibition of egg hatching after 48 h exposure of *H. contortus* to different concentrations of plant extracts (mg/ml) and Albendazole (µg/ml).

Plant type	Mean ± SE at different concentrations						
	0.0	**0.0625**	**0.125**	**0.25**	**0.5**	**1**	**2**
Albendazole	0.29±0.5	15.7±1.4	63.2±1.9	100±0	100±0	100±0	100±0
Carissa spinarum	6.1±0.61	12.5±1.7	36.6±10.2	99.7±0.5	100±0	100±0	100±0
Phytolacca dodecandra	0.61±0.6	2.8±0.12	16.4±1.6	36.8±3.7	72.5±0.5	99.4±0.6	99.4±0.6
Acacia tortilis	0.29±0.9	5.8±0.97	39.4±1.3	68.1±2.5	86.8±2.8	99.7±0.3	100±0
Azadrichta indica	0.61±0.6	4.9±2.6	14.3±1.2	73.2±1.8	98.8±0.8	100±0	100±0

Table 2. Mean percentage mortality of adult parasites after 24 h exposure of *H. contortus* to different concentrations of plant extracts and Albendazole (mg/ml).

Control	Mean ± SE at different concentrations					
	0.0625	**0.125**	**0.25**	**0.5**	1	**2**
Albendazole	37.8±2.2	67.6±3.9	89.9±0.6	100±0	100±0	100±0

Plants	Mean ± SE at different concentrations						
	0.0	**0.25**	**0.5**	**1.0**	**2.0**	**4.0**	**8.0**
Carissa spinarum	21.8±2.8	21.7±1.7	62.1±6.6	89.6±3.3	89.6±0.37	96.8±3.3	100±0
Phytolacca dodecandra	9.8±o.6	26.7±3.3	38.8±10.2	5.8±0.97	39.4±1.3	68.1±2.5	86.8±2.8
Acacia tortilis	16.1±3.1	19.4±0.6	25.6±2.9	4.9±2.6	14.3±1.2	53.03±1.5	51.9±1.9
Azadrichta indica	36.6±	24.1±2.1	36.6±1.9	89.9±3.3	78.8±3.3	93.9±3.1	100±0

fruits of *P. dodecandra* are traditionally used against endo-parasites (Belay, 2004) and the leaves as antiseptic (Haylessielassie, 2004). This is evident from the current study which showed 99.4% inhibition of egg hatchability by the plant extract at a concentration of 0.1 mg/ml and 68.1% of adult mortality of *H. contortus* at concentration of 4 mg/ml. The root, bark and inner bark of *A. tortilis* were reported to treat diarrhea (Sory, 1999; Beyecha, 2004) and the bark was used topically to treat ring worm (Hayatu, 2003).

In the current study, aqueous extract of *A. tortilis* exhibited 99.7% inhibition of egg hatchability at 0.1 mg/ml and 53.03% of adult mortality of *H. contortus* at concentration of 4 mg/ml. Based on the ability of plant extract to inhibit egg hatchability, the most potent extracts in a decreasing order were that of *C. spinarum*, *A. indica*, *A. tortilis* and *P. dodecandra*. And based on the ability of adult mortality, the most potent extracts were that of *C. spinarum*, *A. indica*, *P. dodecandra* and *A. tortilis*. In this study, all the four extracts showed very good effect (72.5 to 100%) in inhibiting egg hatchability at concentrations between 0.5 and 2 mg/ml. Whereas extracts of two (*C. spinarum* and *A. indica*) out of the four medicinal plants showed a very good adult mortality (89.6 to 100%) of *H. contortus* at concentrations between 1 and 8 mg/ml. On the other hand, *A. tortilis* showed weak effect on adult mortality of *H. contortus* at all concentrations used in the study.

Conclusion

In the current study, extracts of all the study plants (*C. spinarum*, *A. indica*, *A. tortilis* and *P. dodecandra*) have shown promising *in vitro* anthelmitic activity against eggs of *H. contortus.* Extracts from *C. spinarum* and *A. indica* have shown promising adult mortality, while extracts of *A. tortilis* did not demonstrate appreciable result. Based on the aforementioned facts, the following recommendations are forwarded: plants that demonstrated promising activities *in vitro* for their different parts in the current study should be further evaluated *in vivo*. Other types of extracts and *in vitro* evaluation must be conducted for those plant parts and extracts not showing promising results. Phytochemical screening and toxicological evaluation should be performed for those that exhibited promising results in the *in vitro* test.

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