INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
14–228/2014/16–6–1199–1203
http://www.fspublishers.org

Full Length Article

In vitro Ovicidal and Wormicidal Activity of Six Medicinal Plants against *Haemonchus contortus*

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Abstract

The present research was conducted to evaluate the anthelmintic activity of six Pakistani plant extracts viz. *Azadirachta indica*, *Adhatoda vasica*, *Nicotiana tabacum*, *Saussuria lappa*, *Terminalia chebula* and *Convolvulus arvensis*. Dried plant material was ground finely in powder and aqueous-methanol extracts were prepared by cold maceration method. *In vitro* anthelmintic activity was evaluated against *Haemonchus contortus* of sheep using adult motility assay (AMA) and egg hatch test (EHT). Varying degree of anthelmintic activity was observed for all the selected plants. In EHT, based on the LC_{50} values, most effective plants (LC_{50} in ppm) in their order of activity were; *N. tabacum* (0.10), *S. lappa* (0.73), *A. indica* (1.73), *C. arvensis* (2.51), *T. chebula* (5.55) and *A. vasica* (15.74). LC_{50} values of three plants viz. *N. tabacum*, *S. lappa* and *A. indica* were statistically similar to LC_{50} of oxfendazole (0.38). In AMA, a time dependent response was observed in all the treatment groups.

Keywords: Neem; Tobacco; Ethno-veterinary; Stomach worm; Anthelmintic; *In vitro*

Introduction

Sheep and goats are major a source of income for poor rural community of Indo-pak subcontinent, but parasitic helminths, especially nematodes of these animals are one of the major constraints to profitable farming. They cause retarded growth, lowered productivity, mortality and high economic losses. In Australia, estimated cost for control of helminthiasis is between 220 to 500 million US$ (McLeod, 1995). In Pakistan losses to sheep and goat industry of Faisalabad (only one city) has been estimated to be Rs. 31.43 million per annum (Iqbal *et al.,* 1993). Synthetic anthelmintics are generally used for the control of gastrointestinal nematodes (GINs) in livestock, but development of resistance in parasites to several anthelmintics (Leathwick *et al.,* 2001; Hamad *et al.,* 2013) and chemical residues and toxicity problems (Kaemmerer and Butenkotter, 1973) have, however, posed a threat in effective chemotherapeutic parasite control program.

These major drawbacks of synthetic anthelmintics have revived the interest in exploiting the potential of medicinal plants for their use as anthelmintics (Iqbal *et al.,* 2012; Zaman *et al.,* 2012; Hamad *et al.,* 2013, 2014). Traditionally plants have been used as anthelmintics, which may, therefore, offer substitute to the use of synthetic chemicals. A large number of plant based anti-parasitic extracts/formulations have been reported for use in human and animals (Said, 1969; Akhtar *et al.,* 2000; Abbas *et al.,* 2012; Iqbal *et al.,* 2012; Sindhu *et al.,* 2012a; Hamad *et al.,* 2013; Lateef *et al.,* 2013; Masood *et al.*, 2013). However, their scientific evaluation is limited as compared to commercial anthelmintics. Some of these plants include; *Artemisia brevifolia* (Iqbal *et al.,* 2004), *Trachyspermum ammi* L. (Lateef *et al.,* 2006), *Butea monosperma* (Iqbal *et al.,* 2006) and *Calotropis procera* (Iqbal *et al.,* 2005). However, there are many more plants, which need to be investigated for their anthelmintic activity using standard parasitological procedures. These plants have been reported by indigenous people for having anti-parasitic activity and are being used in ethno-veterinary system of Pakistan (Sindhu *et al.,* 2012b). Some of these previously mentioned plants have been selected for this study, aiming to evaluate the anthelmintic activity of crude aqueous-methanol extracts. These plants included; *Azadirachta indica* A. Juss (seed), *Adhatoda vasica* L. (leaf), *Nicotiana tabacum* L. (leaf), *Saussuria lappa* DC. (root), *Terminalia chebula* Retz. (bark) and *Convolvulus arvensis* L. (leaf).

Materials and Methods

Present study was conducted at Chemotherapy laboratory, Department of Veterinary Parasitology, University of Agriculture, Faisalabad-Pakistan (UAF) and all the experiments were repeated thrice.

To cite this paper: Sindhu, Z.U.D., Z. Iqbal, M. Asim, A. Ahmad, R.Z. Abbas and B. Aslam, 2014. *In vitro* ovicidal and wormicidal activity of six medicinal plants against *Haemonchus contortus*. *Int. J. Agric. Biol.*, 16: 1199–1203

Preparation of Plant Material

Seed kernels of *A. indica*, *N. tabacum* (leaf), *S. lappa* (root) and *T. chebula* (bark) were procured from local market of Faisalabad, while *A. vasica* (leaf) and *C. arvensis* (leaf) were collected from field. The plant materials were got authenticated from a botanist at UAF. All the plant parts were shade dried and ground into a fine powder in an electric mill. Crude aqueous-methanol extract (CE) was prepared by cold maceration method using 70% aqueous-methanol as previously described by Sindhu *et al.* (2012a). Prepared CEs were refrigerated till further use.

***In vitro* Anthelmintic Activity**

The *in vitro* anthelmintic activity of CE of six plants was evaluated by observing inhibitory effects on hatching of eggs in egg hatch test (EHT) and by evaluating their ability to kill adult worms in Adult Motility Assay (AMT).

Egg Hatch Test

For isolation of eggs, freshly collected adult female *H. contortus* were triturated in phosphate buffer saline (PBS). The suspension was filtered through sieve and then filtrate was centrifuged for 2 min. at about 300 × *g* and sediment was retained. This sediment was re-suspended in saturated solution of sodium chloride to form a convex meniscus above the test tube. After putting a coverslip above test tube sample was centrifuged again using above mentioned conditions. Cover slip was carefully removed and eggs were washed into another test tube and eggs were collected from sediment after centrifugation of this solution. Eggs were washed thrice with distilled water and adjusted to a concentration of 100-200 eggs/mL, using the McMaster technique (Soulsby, 1982).

Bioassay was performed following the technique of Coles *et al.* (1992). A stock solution of desired concentration (w/v) in PBS was prepared from CE and subsequently five 10-fold dilutions were prepared. One mL of each dilution was added in a well of 24 well titration plate, containing 100-200 freshly collected eggs/well. Positive control wells received the different concentrations of oxfendazole in place of plant extracts, while negative control well only received PBS and the egg solution. These titration plates were incubated at 27°C for 48 h. At end of incubation period a drop of Lugol's iodine was added into each well to stop the reaction and number of eggs and first stage larvae (L_1) were counted.

Adult Motility Assay (AMA)

Adult motility assay was conducted on mature *H. contortus* worms, collected from abomasum of freshly slaughtered sheep, following the technique of Sharma *et al.* (1971). Ten worms were exposed in triplicate to each of the following treatments in separate Petri dishes at room temperature (25–30°C):

1. Crude aqueous-methanol extract @ 1.0, 0.5, 0.25, 0.125 and 0.0625 mg/mL (five different concentrations prepared in PBS).
2. Levamisol @ 0.55 mg/mL.
3. PBS (Negative control).

The inhibition of motility of worms was used as indication of worm mortality or paralysis. Motility of worms was observed on different time intervals till seven hours Post treatment (PT) and on every observation motile worms were counted. Worms not showing any motility were picked out and were kept in lukewarm PBS for 10 minutes and in case of revival in motility, the observed worms were counted as alive, otherwise dead.

Statistical Analysis

The data obtained from AMA was analyzed with one-way ANOVA and Tukey HSD using Statistica Version 6 (Stat Soft, Inc., 2001). Results of egg hatch test were analyzed by probit test using "PoloPlus" (LeOra software, 2002) and lethal concentrations were calculated.

Results

Six indigenous plants; *A. indica*, *A. vasica*, *N. tabacum*, *S. lappa*, *T. chebula* and *C. arvensis* were selected for the evaluation of their anthelmintic activity *in vitro*. In EHT a dose dependent response with all the candidate anthelmintics was observed (Fig. 1). Lethal concentration (LC) estimates of different CEs and oxfendazole have been presented in Table 1. Based on the LC_{50} values most effective plants (LC_{50} in ppm) in their order of activity were; *N. tabacum* (0.10), *S. lappa* (0.73), *A. indica* (1.73), *C. arvensis* (2.51), *T. chebula* (5.55) and *A. vasica* (15.74). Crude extract of *N. tabacum* was even found to be more lethal than oxfendazole. LC_{50} of *N. tabacum* (0.10) was less than that calculated for oxfendazole (0.38), though statistically this difference was non-significant ($P>0.05$). LC_{50} values of three plants viz. *N. tabacum* (0.10), *S. lappa* (0.73) and *A. indica* (1.73) were statistically similar to LC_{50} of oxfendazole (0.38), suggesting that these plant extracts have very strong ovicidal activity. While estimated LC_{50} values of *C. arvensis* (2.51), *T. chebula* (5.55) and *A. vasica* (15.74) were significantly higher ($P<0.05$) than our reference ovicidal anthelmintic that is oxfendazole (0.38).

In AMA, a time dependent response was observed in all the treatment groups (Fig. 2). Crude extract of *N. tabacum* showed a dose dependent anthelmintic effect. *N. tabacum* was found as effective as levamisole at the rate of 0.55 mg/mL with 100% death rate, 3 h PT.

The onset of activity with *N. tabacum* was, however, rapid than levamisole (Fig. 2A). But in worms treated with different concentrations of CE of *A. vesica* surprisingly

Table 1: Comparison of the lethal concentration estimates of different plant extracts evaluated for inhibitory effects on hatching of *Haemonchus contortus* eggs, *in vitro*

	Adhatoda vasica	*Azadirachta indica*	*Convolvulus arvensis*	*Nicotiana tabacum*	*Saussuria lappa*	*Terminalia chebula*	Oxfendazole
Slope (SE)	0.367 (0.033)	0.390 (0.031)	0.379 (0.030)	0.539 (0.029)	0.433 (0.029)	0.331 (0.027)	0.725 (0.055)
χ^2	38.733	20.766	26.281	87.294	39.362	30.442	27.286
LC_{30} ppm (95% CI)	0.588 (0.1-2.8)	0.078 (0.0-0.2)	0.104 (0.0-0.3)	0.010 (0.002-0.035)	0.045 (0.011-0.139)	0.145 (0.033-0.551)	0.072 (0.020-0.170)
LC_{50} ppm (95% CI)	15.74 (3.30-156.23)	1.73 (0.65-5.12)	2.51 (0.83-9.35)	0.10 (0.03-0.33)	0.73 (0.24-2.50)	5.55 (1.40-34.25)	0.38 (0.16-0.74)
LC_{80} ppm (95% CI)	3077.8 (269.2-284979.7)	249.6 (61.3-1864.4)	419.2 (80.3-5028.2)	3.5 (0.9-28.1)	64.6 (14.8-586.5)	1926.9 (215.0-68431.9)	5.5 (3.0-11.1)
LC_{90} ppm (95% CI)	48512 (2315-16761255)	3352 (562-47804)	6080 (755-156114)	23 (4-357)	671 (106-12161)	41024 (2590-4203619)	22 (11-59)

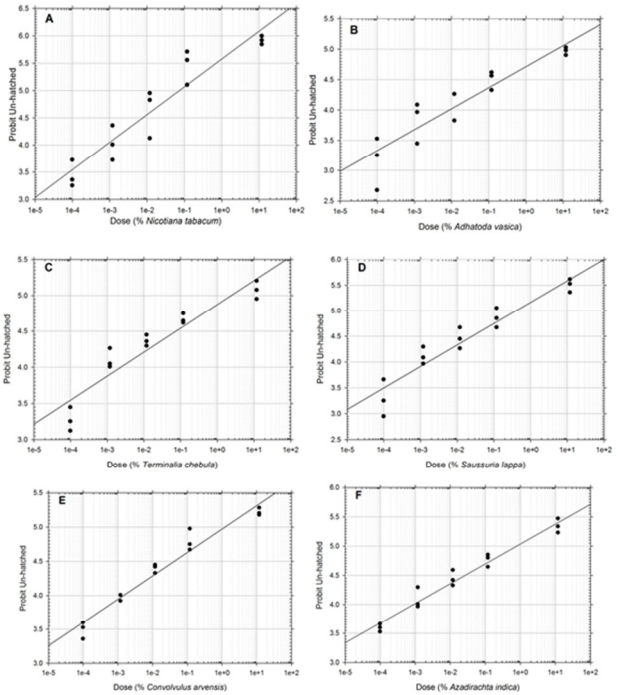

Fig. 1: Probit un-hatched x log concentration plot: eggs of *Haemonchus contortus* submitted to egg hatch test with aqueous-methanol extract of *Nicotiana tabacum* (A), *Adhatoda vesica* (B), *Terminalia chebula* (C), *Saussuria lappa* (D), *Convolvulus arvenses* (E) and *Azadirachta indica* (F) diluted in PBS

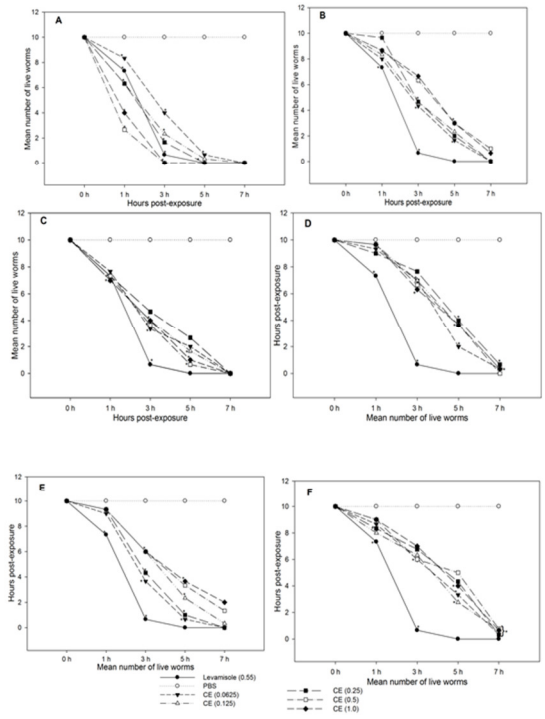

Fig. 2: Graph showing the time-dependent *in vitro* anthelmintic activity of *Nicotiana tabacum* (A), *Adhatoda vesica* (B), *Terminalia chebula* (C), *Saussuria lappa* (D), *Convolvulus arvenses* (E) and *Azadirachta indica* (F) crude aqueous-methanol extract (CE) at 0.0625-1.0 (mg/mL) in comparison with positive control levamisole (0.55 mg/mL), on mature live *Haemonchus contortus* of sheep. The inhibition of motility and/or mortality of the worms were used as the criterion for anthelmintic activity. Values shown are means, asterisk (*) indicates significant difference from previous value at P<0.05. PBS was used as sham treatment

lower doses of extract were found more effective against worms as compared to higher doses of same effect. However, in all the treatment groups a significant effect (P<0.05) was observed on motility/survival of adult *H. contortus* worms. While in PBS, no dead worm was found up to 7 h PT. Although the onset of activity of CE was very slow in comparison with levamisole but 100% worms were found dead in group treated with CE at 0.0625 mg/ml, 7 h PT (Fig. 2E). CE of *T. chebula* significantly (P<0.05) effected the survival of adult *H. contortus* in all the test dilutions (Table 2C). Similarly CEs of *S. lappa, C. arvenses* and *A. indica* also showed a time dependent response. But in all these plants onset of activity was slow as compared with

levamisole, though one or other concentration of all these three CEs killed more than 90% worms at 7 h PT.

Discussion

Ethnoveterinary medicine (EVM) is defined as the medicine that is used by the local farmers to treat the diseases of both human and animal patients using all resources, other than modern synthetic drugs. In developing countries people adopt traditional ways for diagnosis, classification and treatment of common animal diseases. Plants included in the present study are known for treatment of different ailments (Babar *et al.,* 2012; Sindhu *et al.,* 2010, 2012b). In this study, EHT and AMA have been employed to scientifically validate the anthelmintic potential of these plants.

The egg hatch test was initially developed for the diagnosis of benzimidazole resistance helminths. This test has, however, also been used for screening of plants and/or other compounds for their anthelmintic activity (Min *et al.,* 2004; Iqbal *et al.,* 2012; Hamad *et al.,* 2013). In this study, EHT was employed on *H. contortus* eggs using 10 fold dilutions of CE of different plants and their activity was compared with oxfendazole (positive control). Based on LC_{50} values, CE of *N. tabacum* was even found to be more lethal than oxfendazole. This high factor could be due to development of high level of oxfendazole resistance in Pakistani worm population, as reported by Hamad *et al.* (2013). *N. tabacum* (tobacco) is a world renowned plant used for its narcotic properties. A large number of alkaloids of tobacco have been identified and their effects on various biological systems have been reported in literature. Different parts of this plant are widely used traditionally for their antiinflammatory, antirheumatic and anthelmintic properties (Nadkarni, 1976).

Costa *et al.* (2008) tested ethyl acetate extract of *A. indica* on *H. contortus* eggs and reported that 50 mg/mL of ethyl acetate extract inhibited egg hatching by 51.31%, but in our study only 1.73 ppm of *A. indica* CE inhibited 50% hatching. This could be due to difference in solvent used for extraction and moreover we use aqueous-methanol extract containing both the lipophilic and hydrophilic chemicals (Tabassam *et al.,* 2008). It could also be concluded from these results that for initial screening purposes aqueous-methanol extract should be preferred because it contains both the organic and inorganic solvents. EHT was found useful in obtaining reliable data as evident from the χ^2 of different plants screened in this study. Therefore, reliability of EHT as a drug/plant screening assay was in support of the earlier workers (Molan *et al.,* 1999, 2000, 2002; Min *et al.,* 2004).

Similarly these plants extracts were also subjected to AMA to evaluate their effect on adult *H. contortus*. Adult motility assay is the most convenient test used for assaying the anthelmintic activity of drugs/plant products. In AMA, worms are exposed to varying concentrations of drugs and observed for their inhibited motility and/or mortality at different intervals. *H. contortus, Ascaris lumbricoides, Teladorsagia cicumcincta* and *Trichostrongylus colubriformis* have been used for the *in vitro* evaluation of *vitro* anthelmintic activity of plant extracts. In this study, *H. contortus* proved to be good test worm, due to its long survival period in untreated control group. By high merit of its longer survival, more numbers of observations were recorded on the motility of worms. In our results a time dependent response was observed in all the treatment groups. Extract of *N. tabacum* was found as effective as levamisole with a quick onset of activity.

Adhatoda vasica, locally known as "Bhaiker", is conventionally used for treatment of fever (Jain, 1965), cough and asthma (Shah and Joshi, 1971), gastrointestinal disorders; diarrhoea, dysentery and colic and various parasitic infections (Chopra *et al.,* 1958; Dastur, 1962; Atta-Ur-Rahman *et al.,* 1986). Similarly, *T. chebula* is used for the treatment of many chronic diseases such as ageing, heart ailments and hepatic diseases, etc. (Jagetia *et al.,* 2002; Kaur *et al.,* 2002, Sabu and Kuttan, 2002). Kumar *et al.* (2008) reported the wounds healing activity of dried fruits of *T. chebula*. The hot water extract of the roots of *S. lappa* have been traditionally used for treatment of asthma (Shah, 1982; Sircar, 1984), inflammations and rheumatism (Shah, 1982; Lechner, 1982). Though, various pharmacological activities of these plants have been reported, but their anthelmintic activity was unknown against *H. contortus.* In this study anthelmintic activity of these plants has also been scientifically validated for control of *H. contortus*.

It may be concluded that plants considered in this study and used in EVM system of Pakistan have a potential to be used as anthelmintics. And it is recommended that further research be carried out on identification of active principles of plants and their mechanism of action. In addition to this, large number of samples of the same plant from different geographic areas should be subjected to experimentation keeping in view the possibility of differences in chemical composition of the same plant having different soil origin.

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(Received 18 March 2014; Accepted 02 may 2014)