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Anthelmintic properties of extracts from *Artemisia* plants against nematodes

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Abstract. *Artemisia* plant genus, natural inhabitant of northern Punjab Pakistan, is well-known for its anthelmintic properties; many *Artemisia* species have not been so far scientifically proved. The aim of this study was to assess in vitro anthelmintic activity of *Artemisia indica* and *Artemisia roxburghiana* against mixed infection of gastrointestinal nematodes in small ruminants. This study is first scientifically proven study on anthelmintic activity of *A. indica* and *A. roxburghiana.* Five different concentrations (50, 25, 12.5, 6.25 and 3.75 mg/mL) accompanied by negative control (PBS) and positive control (albendazole, 10%) were used to carry out the egg hatch inhibition assay, larval mortality assay and adult worm mortality assay. The Baermann technique was used first time in larval mortality assay and proved to be effective. The results revealed that methanolic extracts of both *A. indica* and *A. roxburghiana*, showed maximum anthelmintic activity at concentration of 50 mg/ml by egg hatch inhibition (85±21.2; 80±28.3), larvae mortality (18±2.8; 17±4.2) and adult worm mortality (8.5±2.1; 8±2.8) assays. However, at concentration of 50 mg/ml both plant extracts in comparison to albendazole showed statistically insignificant ($p \geq 0.05$) results. The *A. indica* showed higher anthelmintic activity at all concentrations as compared to *A. roburghiana*. It has been concluded both plants exhibit anthelmintic activity and further evaluation of these plants should be carried out to purify the active ingredients for anthelmintic activity. Moreover, the decoctions of these plants could be used to GINs after confirming anthelmintic properties through *in vivo*.

INTRODUCTION

Gastrointestinal nematodes cause huge economic losses in small ruminants production worldwide (Cala *et al.*, 2012), mostly invade digestive tract, abomasums and small intestines (Maphosa & Masika, 2009). These parasites obtained nutrition and shelter from their animal host and resulted in poor growth rate, reduced fertility, less immunity, damaged gastric function, high mortality and increased costs of management (Cala *et al.*, 2012). They infect all type of livestock, varies in host range, life cycle and severity. However, nematodiases causes 28% mortality of small stock and weight loss in 3-8 % of livestock, causing US$ 2 billion per annum in many countries (Van Wyk *et al.*, 1999; Zarlenga *et al.*, 2001; Eguale *et al.*, 2007).

Mostly, nematode control is relayed on regular use of commercially available anthelmintics (Molefe *et al.*, 2012). However, repeated use of anthelmintics predisposed nematode populations to become resistant and thus redundant their application

(Carvalho *et al.*, 2012; Hernandez-Villegas *et al.*, 2012; Kotze *et al.*, 2005). Livestock is getting much susceptible to nematode infections due to anthelmintic resistance (Hunter, 1996). As a consequence of anthelmintic resistance, considerable research efforts have been focused on the search of alternative approaches to control the gastrointestinal nematodes in small ruminants (Sutherland *et al.*, 2003; Hughes *et al.*, 2004; Kaplan, 2004; Sargison *et al.*, 2004; Coles, 2005; Hughes *et al.*, 2005; Le Jambre *et al.*, 2005). Many other factors including cost of these anthelmintics and lack of clinical veterinary services in developing countries led to the proposal of screening medicinal plants to treat parasitic infections (Eguale *et al.*, 2011; Fajmi & Taiwo, 2005). It has already been reported that more than 80% of the population in developing countries depend on ethno-veterinary plants for medicinal needs (Fyhrquist *et al.*, 2002). A number of ethno-veterinary plants have been studied for their anthelmintic activities (Botura *et al.*, 2011; Silveira *et al.*, 2012; Cala *et al.*, 2012).

Artemisia plant genus, a member of the family Asteraceae, is well-known for anthelmintic properties (Iqbal *et al.*, 2004). In Pakistan 38 species of *Artemisia* (Asteraceae) has been reported mainly from arid and semiarid areas of Baluchistan, KPK, Northern Punjab and Kashmir (Ghafoor, 2002). However, anthelmintic activity of several *Artemisia* species has not so far been scientifically proved. The present study was, therefore, carried out to assess the anthelmintic activity of *Artemisia indica* and *A. roxburghiana* against mixed infection of gastrointestinal nematodes in small ruminants by using egg hatch inhibition test, larval mortality assay and adult worm mortality test.

MATERIALS AND METHODS

Plant Material Collection and Extraction
In current investigation two *Artemisia* species (*A. indica and A. roxburghiana*) were selected based on the information given by the inhabitants of northern area because of possessing anthelminthic properties. The whole plants containing vegetative shoot o f *A. indica* and *A. roxburghiana* were collected from northern Himalayan region of Pakistan. Both plants were identified, authenticated by a plant taxonomist and voucher specimens were stored in the National Herbarium of the Department of Plant Sciences, Quaid-i-Azam University, Islamabad, Pakistan. The material was completely air dried, crushed and kept in amber coloured bottle until processed.

Methanol Extracts Preparation
Powdered plant material was exhaustively extracted with methanol and crude methanol extract (CME) was evaporated to dryness and stored in an air tight glass bottle at 4 °C until used.

Parasite Eggs And Adults Collection
The eggs collection was made from sheep and goats grazed in Pothwar region, Pakistan. Briefly faeces were recovered directly from the rectum of sheep and goats using two finger procedures. The collection of adult parasites *Haemonchus contortus* and *Telodorsagia* spp. were made from the abomasums of infected sheep and goats slaughtered at local abattoirs. The larvae collection was done by culturing the faeces of sheep and goats.

***In Vitro* Anthelmintic Activity**
In vitro anthelmintic activity of methanolic extracts of genus *Artemisia* plants indigenous to northern areas of Pakistan was carried out by applying the guideline of World Association for the Advancement of Veterinary Parasitology (WAAVP) adopted by Coles *et al.* (1992), Iqbal *et al.* (2004) and Eguale *et al.* (2007) were used with certain modification.

Experimental Design
The *in vitro* anthelmintic activity of extracts was tested in order to assess i) egg hatch inhabitation, ii) larval mortality iii) and adult worm mortality against mixed infection of gastrointestinal nematodes. Five different

concentrations (50, 25, 12.5, 6.25 and 3.75 mg/ml) of the methanolic extracts of *A. indica* and *A. roxburghiana* prepared in Dimethyl sulfoxide (DMSO) and dissolved in Phosphate Buffer Saline (PBS), 10% Albendazole (positive control), and PBS (negative control) were used in the study. The experiment was replicated three times for each concentration. The details of experimental procedure used during the present study are as under:

Egg hatch assay

After egg collection they were mixed with tap water and filtered through different size sieves. For cleaning organic debris mesh size 1 mm and 100 µm was used and eggs were collected on 20 µm. Fresh eggs obtained were washed repeatedly with distilled water. Five different concentrations of methanolic extracts of *A. indica* and *A. roxburghiana* were used as the test treatment. A chemical anthelmintic (Albendazole) was used as a positive control while untreated eggs in Phosphate Buffer Saline (PBS) were kept as negative control. One millilitre of water containing nematode eggs (approximately 100) was placed in each test tube and then a fixed amount (1 mL) of each concentration of methanolic extracts and control was added. The test tubes were covered and kept in an incubator at 27 °C for 48 h. At the end of the trial Lugol iodine solution was added to stop any further hatching. Hatched first stage larvae L_1 (dead or alive) and un-hatched eggs were then counted under dissecting microscope with 40 × magnification.

Larval mortality assay

In order to obtain larvae the freshly collected faeces were pooled and crushed in mortar to make crumbly moist solution. Then an equal amount of vermiculite was added and placed in glass beakers. The beakers were covered with perforated aluminium foil having 10-13 small sized holes for aeration and left for incubation at room temperature for 12-14 days. Subsequently faecal material was poured on cheese cloth present over Baermann apparatus for the collection of L_3 larvae. After 24 hours stages of the L3 larvae were collected from the test tubes attached at the end of Baermann apparatus. These larvae were centrifuged (4000 rpm; 1790xg for 5 minutes) in order to concentrate them and later on they were picked with micropipette and washed in PBS. Then from these washed sheathed larvae, twenty actively moving larvae were selected with the help of pipette and placed in the 24 well flat bottom culture plates filled with each concentration of *A. indica* and *A. roxburghiana* methanolic extracts and control in a total volume of 4 mL. The same concentrations of Albendazole 10% (Pfizer®) were used as a positive control. After 24 h, the extracts and positive control used were washed away and the larvae were suspended in PBS for 30 min for possible recovery of the parasite motility. The number of motile (alive) and immotile (dead) larvae were counted under dissection microscope, and recorded for each concentration. Death or paralysis of larvae was monitored by absence of motility for an observation period of 5–6 seconds.

Adult worm mortality assay

The two freshly collect nematodes species namely *Haemonchus contortus* and *Telodorsagia* spp. were immediately transferred to the laboratory in Ringer solution. Then these worms were picked one by one and washed in the PBS for thirty minutes. Then from these ten actively moving worms were selected and placed in Petri dishes filled with each concentration of *A. indica* and *A. roxburghiana* methanolic extracts and control in a total volume of 5 mL. After 24 h, the extracts and positive control used were washed away and the worms were suspended in PBS for 30 minutes for possible recovery of the worms motility. The number of motile (alive) and immotile (dead) worms were counted under dissecting microscope, and recorded for each concentration. Death or paralysis of worms was ascertained by absence of motility for an observation period of 5–6 seconds as well as also by touching the worms.

Statistical Analysis

Comparison of mean percentages of inhibition of egg hatch, larval and adult parasites mortality at five different concentrations with the control was performed by one-way ANOVA. All the data was analyzed by using the MS Excel and M-Stat C software. Data were expressed as mean ± Standard deviation.

RESULTS

Egg hatch assay

The results indicated anthelmintic activity of both plants *A. indica* and *A. roburghiana*, which induced significant ($p<0.05$) egg hatching inhibition in a dose-dependent manner at a concentration of 25 mg/ml. On the other hand, statistically non-significant ($p>0.05$) egg hatching inhibition was observed (Table 1). Methanolic extract of *A. roxburghiana* induced 80% inhibition at 50mg/ml whereas, the methanolic extract of *A. indica* required a maximum concentration of 25mg/ml, to induce 80% egg hatch inhibition. However, statistically non-significant ($p>0.05$) egg hatch inhibition was observed between *A. indica*, *A. roxburghiana* and reference group (albendazole) at concentration of 50 mg/ml. The insignificant ($p>0.05$) ovicidal activity of PBS among three treatments were observed. Mean percentage inhibition of egg hatch for three drugs at five different concentrations is given in Figure 1.

Larval mortality assay

The methanolic extracts of *A. indica* and *A. roburghiana* cause significant larval mortality in a dose-dependent manner (Table 2). The results indicated a statistically significant ($p\leq0.05$) difference among anthelmintic activity of both plant species, *A. indica* showed higher larval mortality at all concentrations than *A. roburghiana.* On the other hand, both plants did not induce significant mortality at all concentrations tested, maximum mortality was obtained at concentration of 50 mg/ml. Significant ($p<0.05$) larval mortality occurred when drugs were tested at concentration of 12.5 mg/ml against nematode larvae with higher larvicidal activity at albendazole followed by *A. indica* and *A. roburghiana.* Similarly significant difference ($p<0.05$) was found among drugs at concentration of 3.75 mg/ml with maximum larval mortality at albendazole followed by *A. roburghiana* and *A. indica.* However, values of both plants were statistically found insignificant ($p>0.05$) to albendazole at 50 mg/ml. The results of PBS were insignificant ($P>0.05$) among three treatments indicating significantly lower values among for all the treatments tested for larval mortality. Mean percentage mortality of larvae for three drugs at five different concentrations is given in Figure 2.

Adult worm mortality assay

After 24 hours of exposure of adult *Haemonchus contortus* and *Telodorsagia* to methanolic extracts of both plants at different concentration, significant and dose-dependent reduction in mortality was observed. The results of adult worm motility/ mortality test are given in Table 3. The *A. indica* showed statistically significant ($p<0.05$) anthelmintics activity at a concentration of 50 mg/ml against nematodes which was however found insignificant ($p>0.05$) with a concentration of 25 mg/ml. The *A. indica* showed statistically significant ($p<0.05$) anthelmintics activity as compared to *A. roburghiana* by maximum killing of adult worms at different concentration levels. On the other hand, *A. indica* indicated statistically insignificant ($p>0.05$) results to albendazole (control) at all concentration levels. *A. indica* and albendazole killed the parasites in a dose-dependent manner and all the worms were dead at a concentration of 50 mg/ml within 24 hours. The anthelmintic activity of *A. roburghiana* was found statistically significant ($p<0.05$) at a concentration of 50 mg/ml against nematodes whereas insignificant ($p>0.05$) at a concentration of 3.75 mg/ml. The results of PBS (negative control) were found statistically insignificant ($P>0.05$) among three treatments. Mean percentage mortality of adult worms for three drugs at five different concentrations is given in Figure 3.

Table 1. *In vitro* anthelmintic activity of methanolic extracts of *Artemisia indica* and *A. roxburghiana* on nematode eggs of small ruminants after 48 hours. The values are shown in Mean±SD

Conc. mg/mL	*A. indica*	*A. roxburghiana*	Albendazole* (10%)
50	85 ± 21.2^{aA}	80 ± 28.3^{aA}	95 ± 7.1^{aA}
25	80 ± 0.0^{abA}	70 ± 14.1^{bAB}	90 ± 14.1^{aAB}
12.5	70 ± 14.1^{bAB}	60 ± 14.1^{bABC}	90 ± 14.1^{aAB}
6.25	65 ± 21.2^{aAB}	55 ± 35.4^{aABC}	80 ± 14.1^{aBC}
3.75	45 ± 35.4^{aBC}	50 ± 14.1^{aBC}	75 ± 7.1^{aC}
Control (PBS)	35 ± 7.1^{aC}	35 ± 7.1^{aC}	35 ± 7.1^{aD}

*Reference group (positive).
Smaller superscripts compare means in rows.
Capital superscript compares means in columns.
Means with different letter differ significantly ($p \leq 0.05$) among each other.

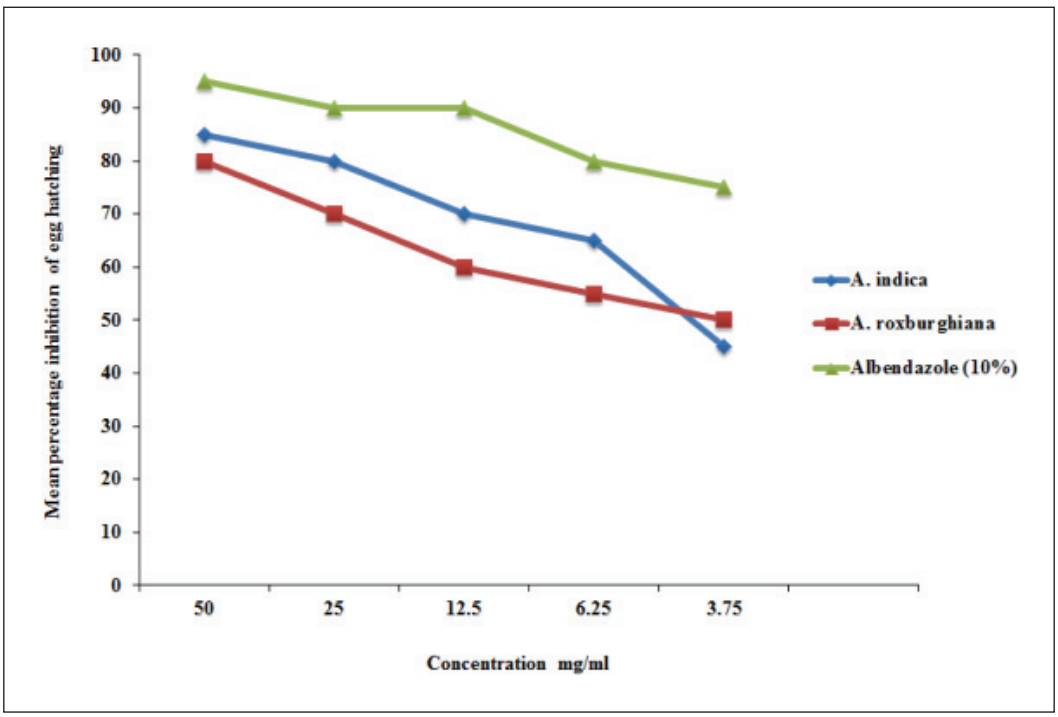

Figure 1. Mean percentage inhibition of egg hatching after 48 hours exposure of eggs of nematodes to five increasing concentrations of both plant extracts and albendazole (positive reference).

Table 2. *In vitro* anthelmintic activity of methanolic extracts of *Artemisia indica* and *A. roxburghiana* on nematode larvae of small ruminants after 24 hours. The values are shown in Mean±SD

Conc. mg/mL	*A. indica*	*A. roxburghiana*	Albendazole* (10%)
50	18 ± 2.8^{aA}	17 ± 4.2^{aA}	19 ± 1.4^{aA}
25	17 ± 1.4^{aAB}	16 ± 2.8^{aAB}	18 ± 2.8^{Aab}
12.5	15 ± 1.4^{bAB}	13 ± 1.4^{cABC}	17 ± 1.4^{Aabc}
6.25	14 ± 2.8^{aB}	12 ± 5.7^{aBC}	16 ± 2.8^{Abc}
3.75	10 ± 5.7^{bC}	11 ± 1.4^{abCD}	15 ± 1.4^{aC}
Control (PBS)	7 ± 1.4^{aC}	7 ± 1.4^{aD}	7 ± 1.4^{aD}

*Reference group (positive).
Smaller superscripts compare means in rows.
Capital superscript compares means in columns.
Means with different letter differ significantly ($p \leq 0.05$) among each other.

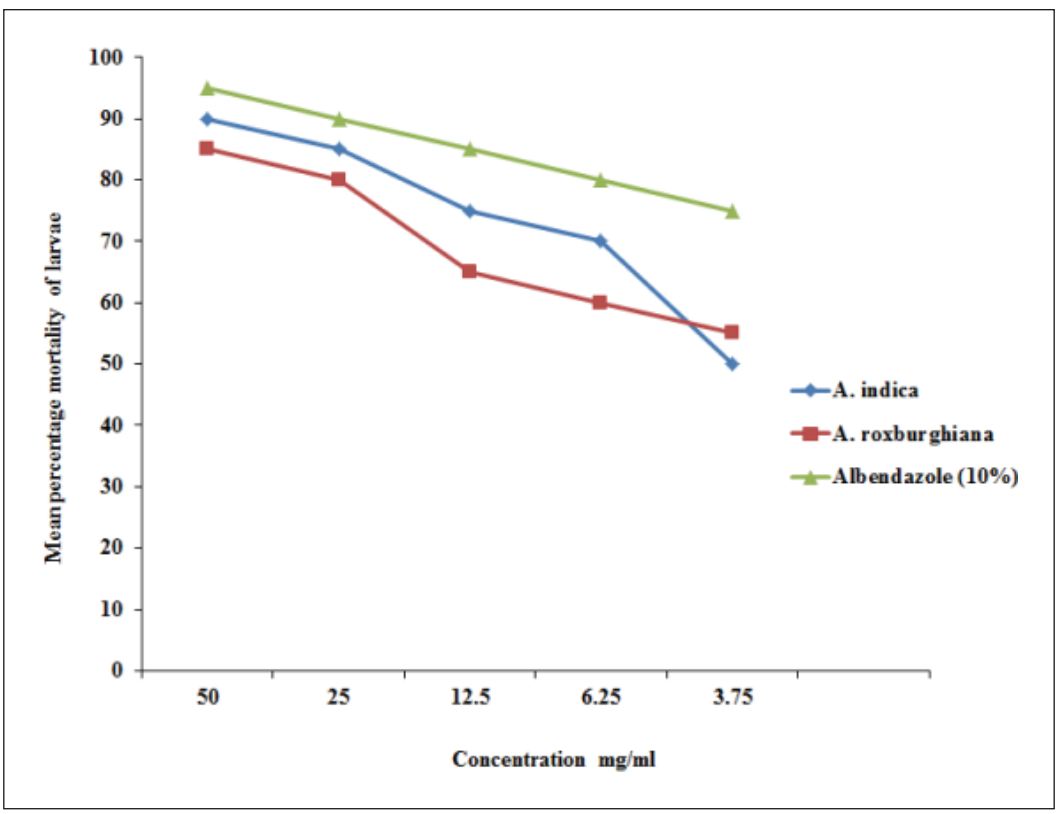

Figure 2. Mean percentage mortality of larvae after 24 hours exposure of larvae of nematodes to five increasing concentrations of both plant extracts and albendazole (positive reference).

Table 3. *In vitro* anthelmintic activity of methanolic extracts of *Artemisia indica* and *A. roxburghiana* on adult nematode worms of small ruminants after 24 hours. The values are shown in Mean±SD

Conc. mg/mL	*A. indica*	*A. roxburghiana*	Albendazole* (10%)
50	8.5 ± 2.1^{aA}	8 ± 2.8^{aA}	9.5 ± 0.7^{aA}
25	8 ± 0.0^{abA}	7 ± 1.4^{bAB}	9 ± 1.4^{aAB}
12.5	7 ± 1.4^{abAB}	6 ± 1.4^{bAB}	8 ± 1.4^{aABC}
6.25	6.5 ± 2.1^{aAB}	5.5 ± 3.5^{aABC}	7.5 ± 0.7^{aBC}
3.75	4.5 ± 3.5^{aBC}	5 ± 1.4^{aBC}	6.5 ± 2.1^{aC}
Control (PBS)	3 ± 1.4^{aC}	3 ± 1.4^{aC}	3 ± 1.4^{aD}

*Reference group (positive).
Smaller superscripts compare means in rows.
Capital superscript compares means in columns.
Means with different letter differ significantly ($p \leq 0.05$) among each other.

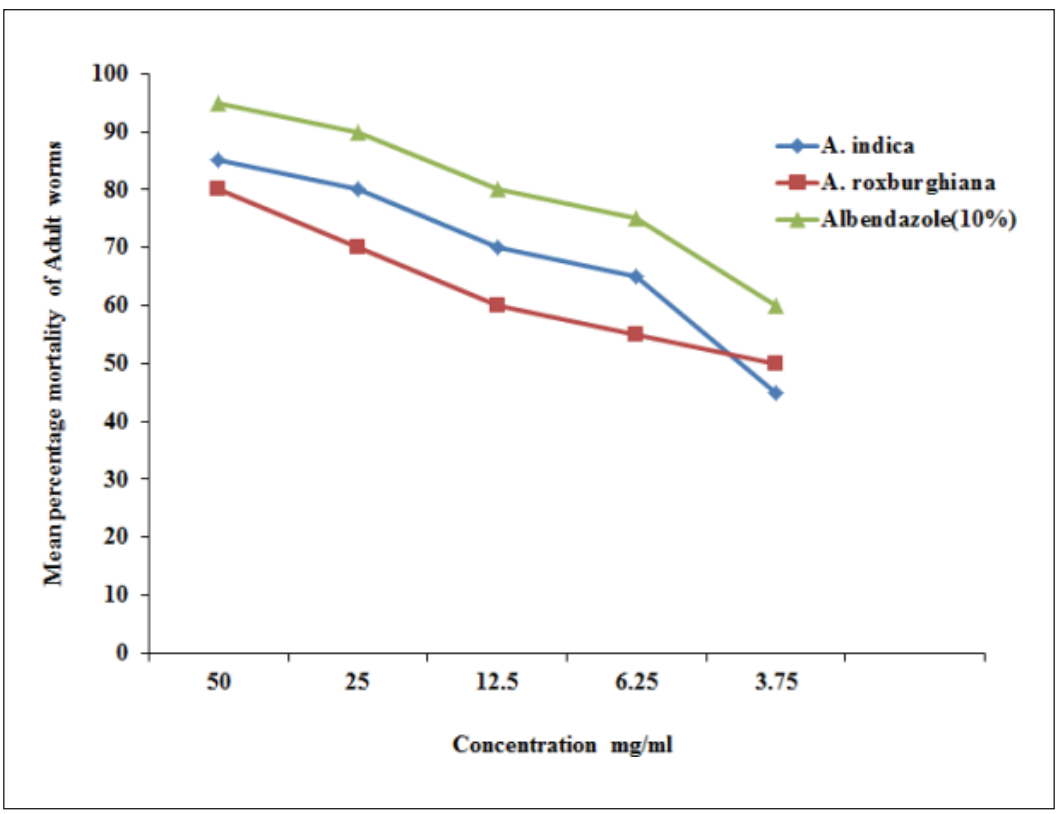

Figure 3. Mean percentage mortality of adult worms after 24 hours exposure of adult nematode worms to five increasing concentrations of both plant extracts and albendazole (positive reference).

DISCUSSION

Plant materials evaluated in the current study had been identified from various sources to serve as anthelmintic agents by traditional healers or farmers in northern Punjab. The genus *Artemisia* plants were selected because of the reported anthelmintic activity in their different species and their parts or products such as oil of flowers *A. pallens* (Naqvi *et al*., 1991; Nakhare & Garg, 1991), *A. herba-alba* (Idris *et al*., 1982), *A. santonica* (El Garhay & Mahmoud, 2002) *A. maritima* (Sharma, 1993) and recently *A. brevifolia* (Iqbal *et al*., 2004). There were only putative reports of the traditional use of these plants for deworming purposes. Our aim was to test local *Artemisia* species for their potential anthelmintic efficacy.

The different concentrations of the plant drugs used in the current study were selected on the basis of the values available in the literature (Assis *et al*., 2003; Maciel *et al*., 2006) in order to save the time for trial. The commercially available albendazole (10%) used in the current study in order to compare *A. indica* and *A. roxburghiana* extracts with the commercially available anthelmintic. Only methanolic extracts of the plants i.e. *A. indica* and *A. roxburghiana* were selected to test (Assis *et al*., 2003; Hördegen *et al*., 2006) because several studies (Okpekon *et al*., 2004; Iqbal *et al*., 2006; Tariq *et al*., 2008) showed that aqueous extracts had very little or no activity against the gastrointestinal nematodes.

Egg hatch inhibition observed in the current study, however, not all eggs were inhibited from hatching and most managed to hatch, this might be because the egg is the stage distributed into the environment and covered with a thick wall making it resistant to various ecological conditions (Hounzangbe-Adote *et al*., 2005). The earlier studies have reported 100% larval mortality which might be due to their much exposure to medicinal components than the eggs (Molan *et al*., 2003). The plants that took part in the current study don't attain 100% larval mortality. The plausible explanation might be chemical constituents which can vary considerably between individual plant species due to genetic or environmental differences, age or developmental stage at harvesting, method of plant material drying, the storage technique and the type of solvent (Hördegen *et al*., 2003; Ononuju and Nzenwa, 2011). Morover, various studies have proven that the ability of eggs and larvae of the gastrointestinal nematodes to withstand at harsh environmental conditions depend on the species and developmental stage of the parasite as well as the geo-ecological regions (Tembley, 1998).

The current results for egg hatch inhibition assay were lower than those reported for Leaf Ether Extract of *Melia azedarach* but quite higher than Leaf Hexane Extracts of same plants (Maciel *et al*., 2006). Jabbar *et al*. (2004) showed an LC_{50} at a lower concentration of *Trachysperum ammi* extracts compared to our results which are yielding efficacy at a higher concentration. The plausible explanation for this difference might be due to difference in experimental conditions, i.e. experimental animals and different plant they used might have stronger anthelmintic compounds than those plants tested in present study. Another possible reason might be the use of single parasite *H. contortus*, while in current study mixed gastrointestinal nematodes were used.

The *A. indica* exhibited a greater larval mortality than those reported in the literature for *Spigelia anthelmia* (Assis *et al*., 2003) when the same concentrations of the plants were used. The probable reason might be use of different plant species and most importantly the method used for acquisition of larvae as the reported author cultured L_1 and L_2 larvae on nutritive media so as to get L_3 larvae, while in current investigation Baermann technique was used. However in present study Baermann technique was used first time to determine the larval mortality assay.

The resultant activity of methanolic extracts of the *A. indica* and *A. roxburghiana* against mixed infection of gastrointestinal nematodes revealed that more than half of the worms of the nematodes were killed at a lower concentration of the methanolic extracts of the plants used i.e. *A. indica* and *A. roxburghiana* in this study i.e.

3.75 mg/mL. However the *A. indica* exhibited a greater activity against mixed infection of nematodes. The current results are much different from those reported by Iqbal *et al.* (2004) who when used the methanolic extract of *A. brevifolia* almost eighty percent of worms were killed within six hours of the incubation with drugs too at a concentration of 25 mg/mL, while our results spanned over a full twenty four hours. Another difference between the two studies is again the use of standard reference. The discussed author used levamisole at a concentration of 0.55 mg/mL and this resulted in mortality of all the worms within six hours at this smaller concentration, while in our case 10% albendazole killed only 95% of the worms at a bigger concentration of 50 mg/mL that too in 24 hours. Similarly in another study, Iqbal *et al.* (2006) reported a higher activity methanolic extract of another plant *Swertia chirata* at a smaller concentration of 25 mg/mL within six hours. However the worm used in both studies were *H. contortus* which is different from our studies we have used the mixed gastrointestinal nematodes acquired from the abomasums of freshly slaughtered naturally infected sheep.

Our results are in agreement with those reported by Eguale *et al.* (2007) who when tested the hydro-alcoholic extracts of *Hedera helix* found a 66.6% mortality at a concentration of 8 mg/mL after an incubation of 24 hours with the plant extracts. However the difference between two studies is that the albendazole used in his case exhibited 100% mortality after 24 hours at a smaller concentration of 0.5 mg/mL compared to the one we used in our study i.e. 50 mg/mL. The reason might be the albendazole used in his study was 99.8% pure, while we used a commercially available 10% albendazole. Same is the case reported by Tariq *et al.* (2008) who evaluated the anthelmintic efficacy of *Iris hookeriana* against sheep nematodes in which 0.55 mg/mL of albendazole yield 100% mortality after eight hours, compared to 80% mortality of *I. hookeriana* in the same time period.

These results revealed the strong and wide spread resistance prevalent among gastrointestinal nematodes of small ruminants of Pothwar region against commercially available anthelmintic i.e. albendazole (10%) which is in agreement with various reports from world over (Taylor *et al.*, 2002; Papadopoulos, 2008). This is an alarming situation and demands strong efforts in order to immediately work out and adopt a multiple strategy to curb this menace.

These *in vitro* studies conducted over gastrointestinal nematodes at egg, larval and mature stages in order to evaluate the *A. indica* and *A. roxburghiana* depicts that these two plants have promising anthelmintic efficacy in them. However activity of *A. indica* was slightly higher than the *A. roxburghiana*. These results are in agreement with those studies who had reported the presence of certain compounds in *Artemisia* such as Artemisinin (Tagboto and Townson, 2001), Santonin (Idris *et al.*, 1982; El Garhay and Mahmoud, 2002). There is a dire need to conduct comprehensive studies using different parts of *Artemisia* spp.to find out the level of anthelmintic activities of these compounds. Besides, other biochemical and pharmacological studies are also required in order to isolate the compounds exhibiting anthelmintic potential in *Artemisia* species.

Artemisia plant species is considered as natural habitant of northern areas of Pakistan where animal husbandry is backbone of the rural sector and development of livestock sector may improve the living standard of rural communities. These animals benefits in term of animal power, hides and supplementary nutrition to poor masses in spite of environmental compulsions and hardship of remote northern areas of Pakistan. Under these circumstances the *Artemisia* spp. could be effectively used for primary health care treatment to make domestic animals productive and healthy. These traditional herbal healers would replace costly, inaccessible and other side effects associated with modern chemical drugs and in turn encouraged the local people to use *Artemisia* spp. as ethano-veterinary therapy for domestic animals.

In vitro egg hatch inhibition test, larval mortality assay and adult worm mortality assay was conducted to confirm the

anthelmintic activity of both *Artemisia spp.* The Baermann technique was used in current investigation for larval mortality assay and proved to be effective. It has been concluded through results that the *Artemisia indica* and *A. roxburghiana* exhibit anthelmintic activity. However, the mechanisms of their effectiveness still remain to be tested *in vivo*. Furthermore, studies on safety and toxicity must be conducted *in vivo* to determine the minimum non-lethal concentrations needed for the treatment of nematode infections.

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