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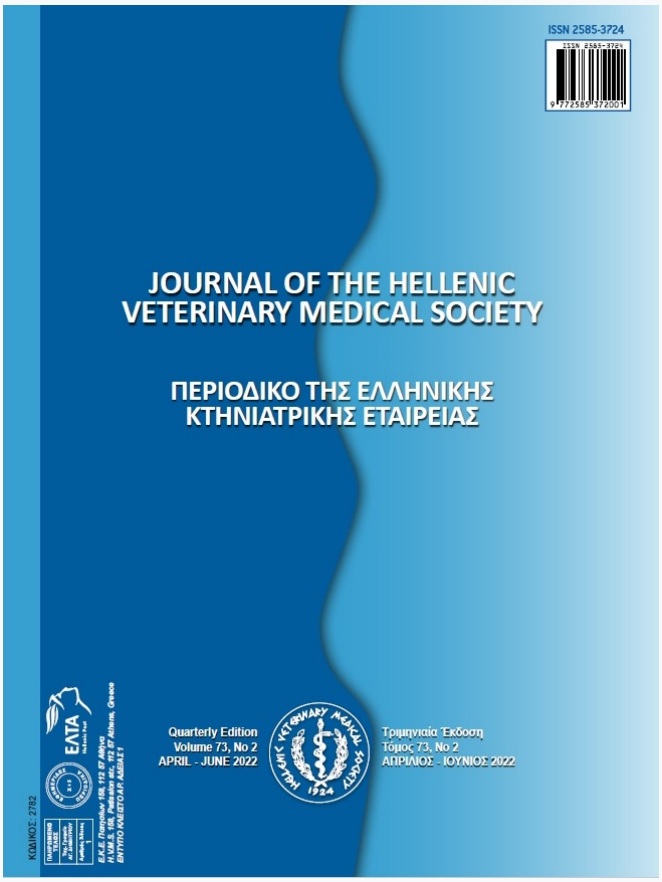

Anthelmintic activity of Moringa oleifera and Azadirachta indica against gastrointestinal nematodes of wild sheep

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Anthelmintic activity of *Moringa oleifera* and *Azadirachta indica* against gastrointestinal nematodes of wild sheep

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ABSTRACT: Gastrointestinal nematodes (GINs) are serious issue for health of wild sheep kept in captivity. Chemically synthesized anthelmintics are regularly used to control these parasites.In recent years anthelmintic resistance and remnant of drugs in animal products leads to use of medicinal plants as alternative to anthelmintics. In current study, the efficacy of aqueous, methanolic and ethanolic dried leaf extracts of medicinal plants *Moringa oleifera* and *Azadirachta indica* were tested for *in-vitro* ovicidal and larvicidal activities against *Haemonchus, Trichuris* and *Trichostrongylus*; naturally acquired nematodes isolated from wild sheep (*Ovisorientalisorientalis*). Six concentrations of these plants extract (1.56, 3.13, 6.25, 12.5, 25 and 50 mg/ml) were evaluated using egg hatching assay (EHA) and larval development assay (LDA) in three replicates. To compare treatment effects, untreated and treated (0.1% ivermectin) controls were used.The aqueous, methanolic and ethanolic leaf extracts showed anthelmintic activities against isolated genera of nematodes but the inhibition was maximum (99%)in ethanol extract of *M. oleifera* followed by methanol extract (97%)at maximum concentration tested at (50mg/ml).The overall findings of this study shows that *Moringa oleifera* and *Azadirachta indica* leaf extracts possess significant anthelmintic efficacy against GINs of sheep and these could be a natural alternative to synthetic anthelmintics to treat the worm infections in animals.

Keywords: Gastrointestinal nematodes; Wild sheep; Anthelmintics; *Moringa oleifera;Azadirachta indica*

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INTRODUCTION

Parasitic diseases, particularly gastrointestinal nematodes (GINs) are among the elements that utmost sheep creation around the world, representing the major financial misfortunes because of decreased sustenance utilization, impeded development, bring down drain generation, weight reduction, disabled ripeness, and in instances of immense contaminations, high death rates (Cavalcante *et al*., 2009; Chinchilla-Carmona *et al*.,2020).

There are distinctive kinds of wild sheep in Pakistan, which are for the most part utilized for ecotourist e.g., Mouflon sheep, Blue sheep, Marcopolo sheep and Big horn sheep (*Ovis canadensis*). Wild sheep (*Ovis orientalis* orientalis) from various secured districts and national parks inspected for gastrointestinal nematodes at necropsy. There are distinctive types of nematodes among wild sheep (Hoberg *et al*., 2008).

Currently, anthelmintics are used for the control of nematodes in the sheep. Factors including drug resistance to anthelmintic (Taylor *et al*., 2009), presence of drug residues in dairy products, awareness at public level and food toxicity needed alternatives techniques and products to control endoparasites. Cost effective and potentially non-hazardous endoparasite control measure is much needed for the farmers to avoid more economical losses (Ademola *et al*., 2010). Traditional medicinal plants as an alternative dewormers proved to be significant in underdeveloped and developed countries. Moreover, use of medicinal plants is a tradition in many countries (Azam *et al*., 2019; Majeed *et al*., 2020; Hassan *et al*., 2020; Adoho *et al*., 2022) and against mosquitoes (Baz *et al*., 2021; Strbac *et al*., 2021). Previous findings related to traditional medicinal plants proved many plant species as a dewormers and alternative to anthelmintics (Abbas *et al*., 2020) that can be helpful to reduce the level of influx of parasites in livestock (Egualee*t al*., 2011; Sobhy*et al*., 2021) that are both sustainable and ecologically acceptable. Abou Laila *et al*. (2021) reported inhibitory effects of Courmermycin A1 on Theileria and Babesia while Ceylan*et al*. (2021) reported the serological changes in vector borne parasitic disease, which highlights the importance of parasitic infestations over the globe.

The derived products from many herbs and plants are source of bioactive compounds and agents that can be used for the treatment of diseases are comparatively harmless for use. Almost 25% synthetic drugs are derived from medicinal herbs and plants, reported by FAO (Food and Agriculture Organization's) and in developing and underdeveloped countries nearly 80% population depends on herbal medicine for the treatment of different diseases (Pan *et al*., 2013; Ragab *et al*., 2020). Although several medicinal plants are already been tested in many countries for their anthelmintic use, some of the scientific reports including Hounzangbe-Adote *et al*. (2005), Alawa*et al*. (2003), Assis *et al*. (2003)and Eguale *et al*., (2007) have proved *Moringa oleifera* Lam. (Sohanjna) as a multipurpose tropical tree. It is also called Drumstick. *Moringa* is a sub-himalayan tree with an average height of 10m (Rastogi *et al*.,2009).

Azadirachta indica (*A. indica*) commonly known as "Neem", is generally utilized for the cure of various animal and human diseases. The most imperative piece of the Neemis the leaf, which has been utilized as leaf powder, crude Neem leaves, Neem leaf extracts and furthermore Neem leaf juice. There are some reports of Neem effects against parasites. The activity of *A. indica* on the parasites was seen in *Hyalomma anatolicum excavatum* and *Bovicola ovis* (Abdel-Shafy and Zayed, 2002). Azadirachtinat 1% stopped 68% of *Haemonchus contortus* egg hatching (Pessoa, 2002).

Due to growing challenge of anthelmintic resistance (AR) against common drugs, there is dire need to explore some natural resources which can replace these compounds due to their therapeutic action against stomach worms. Therefore, this study was carried out to evaluate the therapeutic importance of *M. oleifera* and *A. indica* against GINs of wild sheep. This report can also pave guidance to wildlife department to enhance the plantation of said plants in wild sheep vicinities.

MATERIALS AND METHODS

Plant collection and extract preparation

M. oleifera and *A.indica* leaves were collected locally from different areas in district Faisalabad, Pakistan. Identification of the plants was done by plant taxonomist at Department of Botany, Government College University Faisalabad (GCUF), Pakistan. Fresh and tender leaves of selected plants were used to make extract in aqueous, methanol and ethanol for analysis. Briefly, Plant leaves and blooms were air dried at room temperature, grinded in electric blender and kept in dry conditions for further proceedings. Aqueous, ethanolic and methanolic extract prepared by mixing dry powder (300 gram) of each plant in

900 ml distilled water, ethanol, methanol at 50°C in electric shaker for 3 hours respectively through Soxhlet apparatus. Solvent was evaporated through rotary evaporator and all extracts were stored at 4°C (Debella, 2002).

Phytochemical analysis of the extracts

Phytochemical analysis of the extracts was accomplished by following Hoste *et al* (2006) methodologies. These tests are based on the addition of specific reagents to aliquots of aqueous, methanolic and ethanolic extracts of leaves and observing of solution colors or precipitate formations. Major secondary metabolites of extracts were screened by following Debella (2002) methodology.

Collection of faecal samples

The fresh faecal samples were collected from the wild sheep also called Mouflon sheep naturally infected with GINsat the Wildlife Park Gatwala Faisalabad at the morning time. Each sample was kept in discrete polythene sack and was transported to the lab under proper sterile conditions , Department of Zoology, GCUF.

Collection of eggs and egg hatching assay

GIN eggs were collected and further cultivated by following the previously reported method (Doligalska *et al*., 2011). Homogenous mixture of faecal sample (10 gram) were made in 50 ml distilled water for eggs collection that followed by sieving and centrifugation at 900xg for 2 minutes. Supernatant was discarded and pellet suspended in a saturated NaCl solution (specific weight 1.2), and centrifuged at 170xg for 2 minutes. Each supernatant containing GINs eggs was collected, and eggs were washed with distilled water in 15 mL tubes after centrifugation at 110xg for 2 min. The supernatant was removed and the sediment was collected. Then, 100 µL of the sediment were collected for McMaster slide counter, the number of eggs was microscopically counted. The assay was performed in 24 well plates as per the method described by Athanasiadou *et al*. (2001).Hatching percentage of each concentration were measured to obtain the effective dose $LC_{50.}$ In helminth's egg faecal culture, aqueous, methanolic and ethanolic extracts were respectively tested with 1% Ivermectin (99.3% pure reference standard, DACA) as a positive control and untreated eggs in water as a negative control were used. All three extracts were serially diluted from stock volume of 0.5 ml in distilled water with water containing eggs in culture wells. 1% Ivermectin in Dimethyl Sulfoxide (DMSO) and distilled water at the concentrations of 0.5, 1, 2, 5, 10, and 20 mg/ml were used. Dead and alive larvae and unhatched eggs were observed under dissecting microscope.

Larval development test

First stage larvae were maintained after the incubation of eggs at 27°C for 24 hours by following the reported method of Costa *et al.,* (2008) with minute modification. Briefly, an aliquot of 1 ml containing 100-120 first stage larvae of nematodes were mixed with 5 g of faeces from a sheep free of gastrointestinal nematodes and 6 serial concentrations of the plant extracts to make total volume of 7ml together with water containing larvae and volume of egg free of faeces (faeces were sterilized to remove any helminth eggs present, completely dried by heating to 70°C and ground to a fine powder.). Ivermectin (1%) was dissolved in distilled water at 6 serial concentrations used as a positive control while eggs in parasite free faeces were used as negative control (Abou Laila *et al*., 2021). All samples were incubated for 7-14 days at room temperature. At the cm^3end of 14^{th} day, the wall of each cup containing the sample was thoroughly rinsed with 10 ml of water to collect the larvae. The experiment was conducted in duplicates for each concentration and replicated three times. Then, one drop of Lugol's iodine solution was added and all stage larvae were counted under dissecting microscope at 40xmagnification.

Statistical analysis

Probit analysis was done by using Minitab software to calculate the LC_{50} of all extracts. Mean percentage comparison of egg inhibition and larval development assay were calculated by one-way ANOVA (Finney, 1971).

RESULTS

Phytochemical analysis of *M. oleifera* and *A. indica* extract

Two locally collected medicinal plant's extracts of *M. oleifera* and *A.indica* has shown significant anthelmintic activity against various genera of gastrointestinal nematodes (GINs) including *Haemonchus, Trichuris* and *Trichostrongylus* isolated form faeces of wild sheep. Phytochemical analysis showed higher contents of saponin and tannin compounds in Moringa ethanol extract (MEE) and Neem methanol extracts (NME).

Egg Hatch Essay (EHA)

The result of aqueous, methanolic and ethanolic extracts of two medicinal experimental plants i.e.,*M. oleifera* and *A. indica* inhibited the egg hatch of nematodes at different concentrations of 1.25-50mg/ml. The maximum concentration required egg hatching inhibition for all types of extracts were 50mg/ml. Egg hatching essay of different solvent extracts of two plants are exhibited in (Table 1, 3). All types of extracts of *M. oleifera* showed 86.5, 87.05 and 100% while extracts of *A. indica* showed 87.5, 99 and 92% egg hatching inhibition. Increasing the concentration of extracts caused a dose-dependent significant ($p<0.05$) decrease in egg hatching. Ivermectin induced 100% egg hatching inhibition at the concentration of0.12mg/ml. There was no effects were recorded for PBS treated control group against nematodes.

Calculation of Lethal concentration 50 (LC50)

The inhibition effect on egg hatching was found in aqueous methanol and ethanol extracts of *M. oleifera*LC_{50}=3.95mg/ml, LC_{50}=2.25mg/ml and LC_{50}=1.89mg/ml, respectively while the inhibition effect on egg hatching was found in aqueous methanol and ethanol extracts of *A. indica* LC_{50}=3.25mg/ml, LC_{50}=1.75mg/ml and LC_{50}=2.35mg/ml, respectively and positive control of Ivermectin inhibition of egg hatching was LC_{50}=0.15mg/ml.While comparing the aqueous, methanolic and ethanolic extracts of *M. oleifera* and *A. indica*, it was observed that ethanolic extract of *M. oleifera* and methanolic extract of *A. indica* was most effective against the parasites of nematodes eggs hatching ($p<0.05$).

Larval development inhibition

The leaf extracts of *M. oleifera* and *A. indica* exhibited larval development inhibition against nematodes. About 99% larval development inhibition was observed in leafy ethanol extracts of *M. oleifera* while 99% leafy methanol extracts of *A. indica* when compared with Ivermectin, the positive control ($p<0.05$) (Table 2, 4). The inhibition effect larval development was found in aqueous, methanol and ethanol extracts of *M. oleifera*LC_{50}=4.15mg/ml, LC_{50}=2.75mg/ml and LC_{50}=1.9mg/ml, respectively while the inhibition effect on egg hatching was found in aqueous methanol and ethanol extracts of *A. indica*LC_{50}=3.35mg/ml, LC_{50}=1.89mg/ml and LC_{50}=2.85mg/ml, respectively and positive control of Ivermectin inhibition of egg hatching was LC_{50}=0.15mg/ml. when comparing the aqueous, methanol and ethanol extracts of *M. oleifera* and *A. indica*, it was observed that ethanol extract

Table 1: LC_{50} and percentage of egg hatching inhibition in various concentrations of different extracts of M. oleifera leaves in wild sheep

Extract in	Dose mg/ ml	Dose ug/ml	Hatch % age Inhibition	Log dose	Probit	Regression	LC_{50}
Aqueous	50	1560	80.50	3.193124	3.96	3.98	3.95mg/ml
	25	3130	75.50	3.495544	4.36	4.39	
	12.5	6250	54.50	3.795880	4.67	4.70	
	6.25	1250	37.70	4.096910	5.10	5.16	
	3.13	25000	26.50	4.397940	5.67	5.71	
	1.25	50000	15.50	4.698970	6.08	6.12	
Methanol	50	1560	87.05	3.193124	3.87	3.98	2.25mg/ml
	25	3130	76.16	3.495544	4.53	4.64	
	12.5	6250	53.06	3.795880	4.87	4.97	
	6.25	1250	45.90	4.096910	5.08	5.14	
	3.13	25000	32.20	4.397940	5.71	5.83	
	1.25	50000	13.20	4.698970	6.13	6.24	
Ethanol	50	1560	100.00	3.193124	3.92	3.97	1.89mg/ml
	25	3130	87.60	3.495544	4.61	4.67	
	12.5	6250	76.50	3.795880	5.10	5.25	
	6.25	1250	54.30	4.096910	5.71	5.84	
	3.13	25000	35.70	4.397940	6.13	6.17	
	1.25	50000	14.50	4.698970	7.33	7.43	
Ivermectin	0.12	1200	100.00	3.079181	7.33	7.26	0.15mg/ml
Untreated controlled			0	0.0		0..0	

Table 2: LC_{50} and percentage of larval development inhibition in various concentration of different extract of M. oleifera leaves in wild sheep

Extract in	Dose mg/ml	Dose ug/ml	Hatch%age inhibition	Log dose	Probit	Regression	LC_{50}
Aqueous	50	1560	77.90	3.193124	3.96	3.91	4.15mg/ml
	25	3130	72.40	3.495544	4.39	4.31	
	12.5	6250	51.70	3.795880	4.72	4.69	
	6.25	1250	39.41	4.096910	5.03	5.11	
	3.13	25000	27.80	4.397940	5.58	5.60	
	1.25	50000	15.70	4.698970	6.13	6.19	
Methanol	50	1560	91.09	3.193124	3.92	3.90	2.75mg/ml
	25	3130	79.86	3.495544	4.56	4.19	
	12.5	6250	59.90	3.795880	4.95	4.91	
	6.25	1250	48.80	4.096910	5.23	5.33	
	3.13	25000	33.33	4.397940	5.81	5.87	
	1.25	50000	14.75	4.698970	6.34	6.46	
Ethanol	50	1560	99.99	3.193124	3.96	3.91	1.9mg/ml
	25	3130	85.90	3.495544	4.67	4.61	
	12.5	6250	77.70	3.795880	5.13	5.10	
	6.25	1250	55.90	4.096910	5.74	5.77	
	3.13	25000	37.80	4.397940	6.04	6.09	
	1.25	50000	15.50	4.698970	7.33	7.43	
Ivermectin	0.12	1200	100.00	3.079181	7.33	7.26	0.75mg/ml
Untreated controlled			0	0.0	0.0	0.0	

Table 3: LC_{50} and percentage of egg hatching inhibition at various concentration of extract of A. indica leaves in wild sheep

Extract in	Dose mg/ ml	Dose ug/ml	Hatch %age	Log dose	Probit	Regression	LC_{50}
Aqueous	50	1560	87.55	3.193124	3.92	3.95	3.25mg/ml
	25	3130	74.20	3.495544	4.77	4.80	
	12.5	6250	64.31	3.795880	5.08	5.11	
	6.25	1250	53.80	4.096910	5.36	5.39	
	3.13	25000	41.00	4.397940	5.64	5.70	
	1.25	50000	14.50	4.698970	6.13	6.17	
Methanol	50	1560	99.99	3.193124	4.01	4.08	1.75mg/ml
	25	3130	91.09	3.495544	4.80	4.85	
	12.5	6250	83.71	3.795880	5.71	5.76	
	6.25	1250	76.90	4.096910	5.95	5.99	
	3.13	25000	42.70	4.397940	6.34	6.37	
	1.25	50000	16.60	4.698970	7.33	7.36	
Ethanol	50	1560	91.50	3.193124	3.87	3.90	2.35mg/ml
	25	3130	76.20	3.495544	4.53	4.57	
	12.5	6250	67.50	3.795880	5.03	5.08	
	6.25	1250	51.00	4.096910	5.44	5.49	
	3.13	25000	32.40	4.397940	5.71	5.80	
	1.25	50000	13.70	4.698970	6.34	6.38	
Ivermectin	0.12	1200	100.00	3.079181	7.33	7.26	0.15mg/ml
Untreated controlled			0	0.0	0.0	0.0	

Table 4: LC_{50} and percentage of larval development inhibition at various concentrations of extracts of A. indica leaves in wild sheep

Extract in	Dose ug/ml	Hatch %age	Log dose	Probit	Regression	Dose ug/ml	LC_{50}
Aqueous	1.25	1560	89.70	3.193124	3.77	3.67	3.35mg/ml
	3.13	3130	71.20	3.495544	4.45	4.54	
	6.25	6250	60.00	3.795880	4.86	4.77	
	12.5	1250	42.50	4.096910	5.25	5.12	
	25	25000	29.70	4.397940	5.55	5.76	
	50	50000	11.50	4.698970	6.23	6.20	
Methanol	1.25	1560	99.00	3.193124	4.08	4.11	1.89mg/ml
	3.13	3130	91.20	3.495544	5.03	5.02	
	6.25	6250	81.76	3.795880	5.58	5.60	
	12.5	1250	72.90	4.096910	5.88	5.98	
	25	25000	51.80	4.397940	6.34	6.44	
	50	50000	18.50	4.698970	7.33	7.23	
Ethanol	1.25	1560	92.08	3.193124	4.01	4.16	2.85mg/ml
	3.13	3130	79.77	3.495544	4.50	4.56	
	6.25	6250	61.80	3.795880	4.97	4.76	
	12.5	1250	49.75	4.096910	5.28	5.31	
	25	25000	31.80	4.397940	5.81	5.89	
	50	50000	16.50	4.698970	6.41	6.43	
Ivermectin	0.12	1200	100.00	3.079181	7.33	7.26	0.75mg/ml
Untreated controlled			0	0.0	0.0	0.0	

of *M. oleifera* and methanol extract of *A. indica* was most effective against the parasites of nematodes larvae ($p<0.05$).

DISCUSSION

The results exhibited that aqueous, methanol and ethanol extracts of two plants *M. oleifera* and *A. indica* have an anthelmintic properties against nematodes of wild sheep suppressing the egg hatching and larval development. The dry leaf ethanolic extracts of *M. oleifera* and methanolic extracts of *A. indica* gave more consistent results in egg hatching and larval developmentof isolated nematodes of wild sheep.

To the author' knowledge, there are no published studies about the anthelmintic effect of experimental plants on nematodes of wild sheep in Pakistan. These plants are known to contain a much amount of alkaloids, flavonoids and saponins and tannins and most necessary groups of secondary metabolites of medical interest. *In-vitro*, the plants extracts were placed with eggs and larvae of parasitic nematodes to evaluate the egg hatching and larval development (Hammond *et al*., 1997; Akhtar *et al*., 2000;Raza*et al*., 2022). In the present study, the leaf ethanol extract of *M. oleifera* and methanol extracts of *A. indica* were the most active in inhibition of egg hatching of nematodes, showing 100 and 99% effectiveness in concentration of 50mg/ml (LC_{50}=1.89mg/ml) and 50mg/ml (LC_{50}=1.75mg/ml), respectively. The results of phytochemical screening showed higher contents of saponin and tannin compounds in Moringa ethanol extract (MEE) and Neem methanol extracts (NME). Some past investigation presumed that saponin mixes in plant extracts have ovicidal and larvicidal activity against parasitic worm (Doligalska *et al*., 2011; Egual*et al*., 2007; Marie-Magdelein*et al*., 2009). Tannin compounds have great impacts against gastrointestinal nematodes directly and indirectly (Athanasiadou *et al*., 2001; Hoste *et al*., 2006; Marie-Magdeleine *et al*., 2009). Ethanol concentration of *M. oleifera* and methanol concentration of *A. indica* indicated most extreme restraint of egg incubating and larval development because the presence of active compounds (alkaloids, flavonoids, saponins and tannins).These bioactive compounds are implicated in some plants for ovicidal and larvicidal activities against helminthes (Akhtar *et al*., 2000). The way in which condensed tannins (CT) influence nematode parasites can be named direct and indirect. The immediate impacts of CT may be intervened through CT-nematode associations influencing physiological elements of gastrointestinal parasites (Nguyen *et al*., 2005). Dense tannins can likewise respond specifically by meddling with egg bring forth

and improvement to infective stage hatchlings (Min and Hart, 2003).

The main result of this work was to demonstrate ovicidal activity of neem extract. Leafy methanol extracts of *A. indica* showed 99%egg hatching inhibition and larval development inhibition. Pessoa (2002) also demonstrated that 10 mg/ml of azadiracht in isolated from neem leaves inhibited 68% of *H. contortus* eggs hatch, while in the present work 3.12 mg/ml of ethanol extract inhibited 97.77% of eggs from hatching. However, the mechanism of the ovicidal action still needs to be explained. The ethanol extract is less effective than the synthetic anthelminthic, as 0.015 mg/ml of Ivermectin inhibited 98.10% of egg hatching. Ivermectin is the pure active substance, while the ethanol and methanol extract contains several chemical compounds, among them the active ingredient with ovicidal action, in small amounts. In general, the extract of a plant has small concentrations of active compounds and a great number of promising properties (Jayaprakasha*et al*., 2001; Rafay *et al*., 2021; Degla *et al*., 2022). Neem's effectiveness against parasites is due to compounds that mimic hormones. This activity interrupts the life cycle of parasites by inhibiting the ability of the parasites to feed and prevent parasite eggs from hatching. Moreover, azadirachtin interferes with the central nervous system of parasite via inhibition of excitatory cholinergic transmission and partly blocks the calcium channel resulting in expulsion parasites from host body (Iqbal*et al*. 2007; Singh *et al*., 2013). The leaves of *A. indica* have been used in folk veterinary medicine as an anthelmintic for ruminants (Jabbar*et al*., 2006). While Vieira *et al*. (2007) observed no anthelmintic effect of Neemat a dosage of 30 g of dried leaves per goat/day given for 5 days. However, variation in the anthelmintic activity of *A. indica* may be attributed to these factors in different forms (Iqbal *et al*., 2007). Another possible factor for the discrepant results could be the different origin of the plant material.

Conclusion

In conclusion, dried leaf extracts of two medicinal plants *M. oleifera* and *A. indica* used in current study revealed anthelmintic activity against gastrointestinal nematodes of wild sheep infected with natural roundworm infections. Phytochemical analysis showed presence of various compounds of saponins and tannins. In future, research can be done on different toxicological assays of these extracts so that these plants can be recommended for organic farming as a natural source to counter worm infections in animals.

CONFLICT OF INTEREST

None declared.

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