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RESEARCH ARTICLE

Evaluation of Anthelmintic Activity of Different Fractions of *Azadirachta indica* A. Juss Seed Extract

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ABSTRACT

This investigation was aimed at determination of anthelmintic activity of different fractions of *Azadirachta indica* seed prepared from the crude aqueous methanol extract (CAME). For this purpose, eggs and adult *Haemonchus contortus* were exposed to aqueous, ethyl acetate, chloroform and petroleum spirit fractions of *A. indica* seeds employing standard bioassays. All the fractions of *A. indica* seeds exhibited dose and/or time dependent ovicidal and wormicidal effects against *H. contortus*. The best ovicidal activity was demonstrated by the ethyl acetate fraction with LC_{50}=21.32 µg/ml; whereas, it was 6-14 times lower for the other fractions. Likewise, ethyl acetate fraction @ 50 mg ml^{-1} exhibited the best wormicidal effects by killing 83% adult *H. contortus* one hour post-exposure. For *in vivo* anthelmintic activity faecal egg count reduction test was perform on sheep naturally infected with helminthes. Maximum reduction (98.9%) was observed with CAME. Therefore, it is concluded that future studies aimed at identification of active anthelmintic principles of *A. indica* may be focused on ethyl acetate fraction of the plant.

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INTRODUCTION

Helminthiasis is among the most common and economically important diseases of livestock, especially of small ruminants in the tropics and subtropics. They result in lowered animal productivity due to reduction in appetite, loss of body weight, meat and wool, hypoproteinemia, impaired digestive efficiency and retarded growth and mortality (Sykes, 1994).

Gastrointestinal nematodes (GINs) infection in sheep and goats is usually controlled with synthetic anthelmintics in combination with grazing and nutritional management, biological control and traditional medicinal practices including botanical dewormers (Alawa *et al*., 2010; Badar *et al*., 2011). One of the problems associated with commonly used synthetic anthelmintics is the development of drug resistance. Significant levels of anthelmintic resistance have been reported in GINs of sheep and goats in Pakistan (Saddiqi *et al*., 2006), which have led to frequent treatment failures.

Keeping in view the problem of resistance, medicinal plants are potential candidates for their use as anthelmintics. Plants have been frequently reported to possess antimicrobial (Karcıoglu *et al.*, 2011; Oly *et al.*, 2011), insecticidal (Fan *et al.*, 2011; Yildirim *et al*., 2012) and anthelmintic activity for treatment of infections in livestock and humans (Hussain *et al.*, 2010; Iqbal *et al*., 2011; Sindhu *et al*., 2010; 2012). Indigenous knowledge of a community forms the basis for identifying medicinal plants and their uses in ethnomedicine. Some Pakistani plants have been found effective against nematodes (Akhtar *et al*., 2000; Iqbal *et al*., 2003) using their crude powder, aqueous, methanolic and aqueous methanolic extracts. Whereas, for more precise studies on the nature of active principles, plant fractions made in solvents of different polarities should be tested for various biological activities. That is why, present study was designed with an aim of making an assessment of the nature of active anthelmintic principles of *A. indica* (neem) by evaluating the anthelmintic efficacy of its fractions prepared in different solvents, i.e., aqueous, ethyl acetate, chloroform and petroleum spirit.

MATERIALS AND METHODS

Preparation of crude extract and fractionation: Neem seed kernels were purchased from the local market of Faisalabad (Pakistan). The seed kernels were cleaned of adulterants and ground to obtain crude powder (CP). Crude aqueous methanol extract (CAME) was prepared by soaking the CP in 70% aqueous-methanol by cold

maceration at least for three days, after which the solution was filtered through two layers of porous cloth, and the plant material was re-soaked twice. At the end of extraction procedure, combined filtrate was evaporated in a rotary evaporator at 40°C under reduced pressure to obtain CAME (Sindhu *et al*., 2012). Different fractions of CAME were prepared by standard phytochemical procedures using three organic solvents (Williamson *et al*., 1998), i.e., ethyl acetate, chloroform and petroleum spirit. CAME was dissolved in distilled water and transferred to a separating funnel. An appropriate quantity of ethyl acetate was then added into the separating funnel. This mixture was shaken vigorously and after about 30 minutes, the ethyl acetate layer was separated after formation of two distinct layers. More ethyl acetate was added and same procedure was repeated until ethyl acetate became transparent. All the layers of ethyl acetate were combined and sodium sulphate was added to remove any dissolved water. The ethyl acetate fraction (EA) was condensed in a rotary evaporator and then condensate was freeze dried. Likewise, chloroform (Ch) and petroleum spirit (PS) fractions were made from the remaining aqueous fraction (Aq) of the CAME. All the dried extracts, its fractions and CP were stored at 4°C until used.

***In vitro* anthelmintic activity:** To evaluate the *in vitro* anthelmintic activity of CAME and its fractions, Adult Motility Assay (AMA) and Egg Hatch Assay (EHA) were performed using *Haemonchus* (*H.*) *contortus* as test organism.

Parasite materials: Adult *H. contortus* were collected, in phosphate buffered saline (PBS), from the abomasum of sheep at necropsy. Male and female worms were collected separately. Some female worms were crushed to liberate eggs, which were used for EHA while the remaining female and male worms were used for AMA.

Adult motility assay: AMA was performed following the method described by Singh *et al*. (1985). Mature worms (n=10) were added in three replicates to each of the following treatments in separate Petri dishes at room temperature (25-30°C).

1. CAME @ 50, 25, 12.5, 6.25, 3.12 and 1.56 mg/ml
2. Ethyl acetate fraction @ 50, 25, 12.5, 6.25, 3.12 and 1.56 mg/ml
3. Chloroform fraction @ 50, 25, 12.5, 6.25, 3.12 and 1.56 mg/ml
4. Petroleum spirit fraction @ 50, 25, 12.5, 6.25, 3.12 and 1.56 mg/ml
5. Levamisol @ 0.55 mg/ml
6. PBS

The inhibition of motility of worms was used as criterion for anthelmintic activity. The motility was observed at 0, 1, 3, 6, 8, 10 and12 hours intervals. Finally, the worms were kept for five minutes in lukewarm fresh PBS for the revival of motility. The number of dead worms was recorded for each treatment.

Egg hatch assay: EHA was performed following the guidelines of "World Association for the Advancement of Veterinary Parasitology" for determining anthelmintic resistance with modifications that allowed testing of the natural products (Alawa *et al.*, 2003). The assay was performed in 24-multiwell plates. In the assay, approximately 250 eggs in 1.5 ml of water were placed in each well. Five concentrations of each plant extract/fraction and oxfendazole (dissolved in 0.5% DMSO) were placed as treatments in the different wells as follows:

- CAME, EA fraction, PS fraction and Ch fraction: 12, 0.12, 0.012, 0.0012, and 0.00012 mg/ml
- Oxfendazole: 25, 2.5, 0.25, 0.025, 0.0025 and 0.0025 µg/ml

Theses plates were incubated at 28°C for 48 hours and then percentage of hatched and unhatched eggs was determined by observing under an inverted microscope.

***In vivo* anthelmintic activity:** *In vivo* anthelmintic activity was evaluated by fecal egg count reduction test (FECRT). For this; 48, 4–8 month old sheep having naturally acquired mixed GINs were selected from Barani Livestock Production Research Institute, Kherimurat, Attock–Pakistan. Prior to treatment, faecal samples were collected from rectum of each animal. On day 0, the sheep were divided in eight groups based on their live weight and egg per gram (EPG) and were treated. Group A remained untreated control; group B was drenched with levamisole @ 0.5 ml/kg b.wt; groups C to E were administered CP @ 1.0, 1.5 and 2.0 g kg^{-1} b.wt; and groups F to H were administered with CAME @ 1.0, 1.5 and 2.0 g kg^{-1} b.wt. After treatment, EPG were performed on each animal on day 5, 10, 14 and 17 post-treatment and percent reduction was calculated (Coles *et al*., 1992).

Statistical analyses: The data from FECRT and adult motility assay were statistically analyzed using SAS software. The results were expressed as mean ± standard error of mean. For EHA, probit analysis was performed to determine the extract concentration required to prevent 50% hatching, i.e., lethal concentration 50 (LC_{50}).

RESULTS

***In vitro* anthelmintic activity**

Adult motility assay: CAME and all of its fractions included in this assay exhibited anthelmintic activity. Some variations, however, were recorded in the anthelmintic effects among different fractions. But with each treatment, a dose and time dependent anthelmintic effect was observed. The results of AMA have been presented in Table 1. Petroleum spirit and aqueous fractions were found least effective as there was only 90% mortality with highest doses of these fractions 12hr PT; while ethyl acetate fraction was found to be the most toxic of all the fractions and CAME. But all the extracts evaluated in this study were less toxic compared to levamisole, a synthetic anthelmintic drug.

Egg hatch assay: CAME and all of its four fractions inhibited egg hatching and, thus, indicated their anthelmintic (ovicidal) effects (Table 2). Ethyl acetate fraction was found to be have most potent ovicidal phytochemicals (LC_{50}=21.32 µg/ml) followed by chloroform fraction (LC_{50}=114.80 µg/ml), CAME (LC_{50}=274.58 µg/ml), aqueous fraction (LC_{50}=326.19 µg/ml) and petroleum spirit fraction (LC_{50}=335.99 µg/ml). The data of correlation of regression (y and R^2 values) also indicated the dose dependent inhibition of egg hatching of all the treatments.

***In vivo* anthelmintic activity:** A graded dose response in EPG reduction was recorded with CP and CAME of *A. indica*. CAME was found more effective than CP against GINs in all the experimental groups (Table 3). CAME and CP showed the slow onset of activity in comparison with the synthetic anthelmintic. Maximum reduction in EPG (98.91%) was observed with CAME administered @ 2g/kg after 14 days of treatment.

DISCUSSION

There are still a large number of pastoralists/farmers who believe that allopathic drugs administered to animals are deleterious to their health. Nevertheless, there is a large number of qualified veterinarians/paraveterinary staff, who advocates the use of ethno-veterinary medicine (EVM) practices other than preventive medication. Therefore, EVM practices have a crucial role in animal health and production in Pakistan. This situation is typical of a rural underdeveloped culture like that of pastoralists of Africa (de Leeuw *et al.*, 1995) and other parts of world having dependence on EVM practices for their animals.

Despite the fact that rural people reject many inventions of modern technology, research on indigenous knowledge and skills indicate that these resources are valuable and could contribute towards development. EVM has been developed by farmers, rather than by scientists in laboratories. Therefore, it is less organized and less formalized, and is usually transferred orally rather than in writing. To date, a large number of plants have been documented during ethnobotanical surveys for their use to kill/repel pests/parasites.

In the present study, *A. indica* seeds demonstrated best anthelmintic activity resulting in 98.9% reduction in EPG by day 14 post-treatment in sheep treated with CAME @ 2 g/kg body weight, 100% *in vitro* mortality of *H. contortus* by 6th hour post-exposure to ethyl acetate fractions @ 50 mg ml^{-1}, and 21.32 µg ml^{-1} LC_{50} recorded in EHA. *A. indica* is also well known as "Divine Tree" and "Village Pharmacy". Medicinal properties of neem include anti-inflammatory, antiviral, antipyretic, immunostimulant, analgesic, diuretic, antimicrobial, antimalarial and anthelmintic properties (Dhawan and Patnaik, 1993). Leaves, fruits, bark, flowers and seeds of *A. indica* have been used for medicinal purpose for centuries in almost every part of the world especially in the Indian subcontinent.

Different parts of *A. indica* have been reported for their antiparasitic use as anthelmintic, acaricide and insecticide (Githiori *et al.*, 2003; Tabassam *et al.*, 2008). Azadirachtin (a compound isolated from neem seeds) has been reported for its inhibitory effects on *H. contortus* egg hatching (Pessoa, 2001). Ali (2004) isolated Aazadirone, epoxyazadiradione, nimbaflavone and azadiradione from n-hexane fraction of the flowers of *A. indica*. Methanolic extract of *A. indica* flowers

Table 1: Effect of CAME of *Azadirachta indica* seed kernels and its fractions on survival of adult *Haemonchus contortus* in comparison with synthetic anthelmintic

Treatments	Mean number of dead worms at different hours						
mg/ml	0 hr	1 hr	3 hr	6 hr	8 hr	10 hr	12 hr
Negative and Positive control							
Lev 0.5	0.0±0.00^{m}	10.0±0.00^{a}	10.0±0.00^{a}	10.0±0.00^{a}	10.0±0.00^{a}	10.0±0.00^{a}	10.0±0.00^{a}
PBS	0.0±0.00^{m}	0.0±0.00^{m}	0.0±0.00^{m}	0.0±0.00^{m}	0.0±0.00^{m}	0.0±0.00^{m}	0.0±0.00^{m}
Crude aqueous methanol extract							
1.56	0.0±0.00^{m}	0.0±0.00^{m}	0.0±0.00^{m}	3.3±0.33^{ij}	5.3±0.33^{fg}	8.0±0.00^{d}	10.0±0.00^{a}
3.12	0.0±0.00^{m}	0.0±0.00^{m}	2.0±0.58^{k}	4.0±0.58^{hi}	6.0±0.00^{f}	10.0±0.00^{a}	10.0±0.00^{a}
6.25	0.0±0.00^{m}	1.0±0.58^{l}	2.7±0.33^{jk}	4.7±0.67^{gh}	7.0±0.58^{e}	10.0±0.00^{a}	10.0±0.00^{a}
12.5	0.0±0.00^{m}	3.0±0.00^{j}	5.3±0.33^{fg}	6.0±0.00^{f}	9.0±0.00^{bc}	10.0±0.00^{a}	10.0±0.00^{a}
25	0.0±0.00^{m}	6.0±0.58^{f}	7.0±0.58^{e}	8.0±0.58^{d}	9.3±0.33^{ab}	9.7±0.33^{ab}	10.0±0.00^{a}
50	0.0±0.00^{m}	7.0±0.58^{e}	7.7±0.33^{de}	8.3±0.33^{cd}	9.7±0.33^{ab}	10.0±0.00^{a}	10.0±0.00^{a}
Ethyl acetate fraction							
1.56	0.0±0.00^{m}	3.3±0.33^{ij}	4.7±0.33^{j}	6.0±0.00^{gh}	7.3±0.33^{ef}	9.0±0.00^{bc}	10.0±0.00^{a}
3.12	0.0±0.00^{m}	4.0±0.58^{k}	5.7±0.33^{hi}	6.3±0.33^{h}	7.7±0.67^{de}	10.0±0.00^{a}	10.0±0.00^{a}
6.25	0.0±0.00^{m}	5.0±0.58^{ij}	6.0±0.00^{gh}	7.3±0.33^{ef}	8.3±0.33^{cd}	10.0±0.00^{a}	10.0±0.00^{a}
12.5	0.0±0.00^{m}	5.7±0.33^{hi}	6.7±0.33^{fg}	8.0±0.00^{de}	9.3±0.33^{ab}	10.0±0.00^{a}	10.0±0.00^{a}
25	0.0±0.00^{m}	6.0±0.58^{gh}	7.3±0.33^{ef}	9.0±0.00^{bc}	9.0±0.00^{bc}	10.0±0.00^{a}	10.0±0.00^{a}
50	0.0±0.00^{m}	8.3±0.33^{d}	9.3±0.33^{ab}	10.0±0.00^{a}	10.0±0.00^{a}	10.0±0.00^{a}	10.0±0.00^{a}
Chloroform fraction							
1.56	0.0±0.00^{p}	0.0±0.00^{p}	0.0±0.00^{p}	2.3±0.33^{o}	4.0±0.00^{m}	6.3±0.33^{hi}	8.7±0.67^{bcd}
3.12	0.0±0.00^{p}	0.0±0.00^{p}	0.0±0.00^{p}	3.0±0.00^{no}	4.3±0.33^{lm}	7.7±0.33^{ef}	10.0±0.00^{a}
6.25	0.0±0.00^{p}	0.0±0.00^{p}	2.3±0.33^{o}	3.7±0.67^{mn}	5.3±0.33^{jk}	7.3±0.33^{fg}	10.0±0.00^{a}
12.5	0.0±0.00^{p}	4.0±0.00^{m}	4.3±0.33^{lm}	5.3±0.33^{jk}	6.7±0.67^{gh}	8.3±0.33^{cde}	10.0±0.00^{a}
25	0.0±0.00^{p}	5.0±0.58^{kl}	6.0±0.00^{hij}	7.3±0.33^{fg}	8.7±0.33^{bcd}	9.0±0.00^{bc}	10.0±0.00^{a}
50	0.0±0.00^{p}	5.7±0.33^{ijk}	6.0±0.58^{hij}	8.0±0.00^{def}	9.0±0.58^{bc}	9.3±0.33^{ab}	10.0±0.00^{a}
Petroleum spirit fraction							
1.56	0.0±0.00^{p}	0.0±0.00^{p}	0.0±0.00^{p}	0.0±0.00^{p}	3.3±0.33^{m}	5.7±0.33^{ij}	6.3±0.33^{ghe}
3.12	0.0±0.00^{p}	0.0±0.00^{p}	0.0±0.00^{p}	1.3±0.33^{o}	5.7±0.67^{ij}	6.7±0.33^{fgh}	7.3±0.33^{def}
6.25	0.0±0.00^{p}	0.0±0.00^{p}	0.0±0.00^{p}	2.3±0.67^{n}	6.0±0.00^{hij}	7.0±0.58^{efg}	8.3±0.33^{bc}
12.5	0.0±0.00^{p}	2.7±0.33^{mn}	4.3±0.33^{l}	4.7±0.67^{kl}	6.3±0.33^{ghi}	7.3±0.33^{def}	9.0±0.00^{b}
25	0.0±0.00^{p}	4.3±0.33^{l}	5.3±0.33^{jk}	6.0±0.00^{hij}	7.3±0.33^{def}	8.0±0.00^{cd}	9.0±0.00^{b}
50	0.0±0.00^{p}	4.7±0.67^{kl}	6.0±0.00^{hij}	7.0±0.00^{efg}	7.7±0.33^{cde}	8.3±0.33^{bc}	9.0±0.00^{b}
Aqueous fraction							
1.56	0.0±0.00^{o}	0.0±0.00^{o}	0.0±0.00^{o}	1.0±0.58^{n}	2.7±0.67^{lm}	4.3±0.33^{ij}	5.0±0.00^{ghi}
3.12	0.0±0.00^{o}	0.0±0.00^{o}	0.0±0.00^{o}	2.0±0.00^{m}	3.3±0.33^{kl}	4.7±0.67^{hi}	5.3±0.33^{fgh}
6.25	0.0±0.00^{o}	0.0±0.00^{o}	0.7±0.67^{no}	2.0±0.58^{m}	5.0±0.00^{ghi}	5.7±0.67^{efg}	6.3±0.33^{de}
12.5	0.0±0.00^{o}	0.0±0.00^{o}	1.0±0.00^{n}	2.3±0.33^{m}	5.3±0.33^{fgh}	6.0±0.00^{ef}	7.0±0.00^{cd}
25	0.0±0.00^{o}	0.0±0.00^{o}	0.3±0.33^{no}	3.7±0.67^{jk}	6.0±0.00^{ef}	6.3±0.33^{de}	7.7±0.67^{c}
50	0.0±0.00^{o}	0.0±0.00^{o}	2.7±0.67^{lm}	4.3±0.33^{ij}	6.3±0.33^{de}	7.7±0.67^{c}	9.0±0.00^{b}

($^{a-p}$) Means sharing at least one same letters in a column or row for each treatment do not differ significantly at P≥0.05.

Table 2: Lethal Concentration$_{50}$ (LC_{50}), regression and R^2 values of CAME of *Azadrichta indiaca* seed kernels and its fraction on hatchability of *Haemonchus contortus* eggs

Plant extracts and fractions	Hatching %					LC_{50} µg/ml	Y	R^2
	Plant extracts/fractions concentrations (µg/ml)							
	1.2	12	120	1200	12000			
Crude aqueous methanol extract	89	82	61	45	11	274.58	-0.5947+8.2344	0.9528
Ethyl acetate fraction	71	55	35	25	9	21.32	-0.4589+6.9863	0.9883
Chloroform fraction	76	61	51	36	18	114.80	-0.3881+6.9639	0.9379
Petroleum spirit fraction	85	77	55	35	29	335.99	-0.4304+7.3784	0.9721
Aqueous fraction	82	71	49	43	31	326.19	-0.3552+6.9586	0.9686

Table 3: Effect of CP and CAME of *Azadirachta indica* on eggs per gram of faeces [Mean+SEM (% reduction)] in sheep

Day	PT[1]	Crude Powder			Crude Aqueous Methanol Extract			Control	
		1.0 g	1.5 g	2.0 g	1.0 g	1.5 g	2.0 g	Untreated[2]	Treated[3]
0		3008.3±174.4^{a}	3016.7±236.2^{a}	2533.3±408.8^{a}	2533.3±374.8^{a}	2725.0±312.2^{a}	3058.3±355.5^{a}	3025.0±324.0^{a}	3333.3±274.1^{a}
5		1508.3±131.3^{b} (49.9)	1208.3±120.0^{b} (59.9)	1008.3±128.1^{b} (60.2)	1275.0±44.3^{b} (49.7)	975.0±108.6^{b} (64.2)	425.0±38.2^{b} (86.1)	3033.3±99.7^{a} (-0.3)	83.3±38.0^{b} (97.5)
10		1175.0±105.5^{b} (60.9)	716.7±76.0^{c} (76.2)	350.0±59.2^{c} (86.2)	600.0±73.0^{c} (76.3)	441.7±89.8^{c} (83.8)	141.7±35.2^{b} (95.4)	3208.3±137.5^{a} (-6.1)	16.7±10.5^{b} (99.5)
14		266.7±57.3^{c} (91.1)	175.0±47.9^{d} (94.2)	125.0±28.1^{c} (95.1)	83.3±21.1^{d} (96.7)	58.3±32.7^{c} (97.9)	33.3±16.7^{b} (98.9)	3225.0±174.5^{a} (-6.6)	0.0±0.0^{b} (100.0)

($^{a-d}$) Means sharing at least one same letters in a column do not differ significantly at P≥0.05; [1]Post treatment; [2]Untreated infected control group; [3]Group treated with Levamisole @ 7.5 mg kg^{-1} b.wt.

was found to contain many chemical compounds like *O*-methylazadironolide, diepoxyazadirol, 3'-prenylnaringenin, isoazadironolide, 1-hydroxy-2-(*p*-hydroxyphenyl) ethane, flowerine and azadironolide (Ali, 2004). Ethanolic extracts of fresh neem leaves are rich in different limonoides and these limonoides showed antimalarial activity (Joshi *et al*., 1998).

The insecticidal properties of *A. indica* are well established and are mainly attributed to Azadirachtin. It has been suggested that azadirachtin interfere with the neuroendocrine system (Bidmon *et al*., 1987). Its exact mechanism of action has not been determined yet, but various hypotheses exist. Rembold *et al*. (1983) suggested that it interferes with the neuroendocrine system and hinders with the production of ecdysone and juvenile hormone. Rembold *et al*. (1983) has also reported that the control by azadirachtin of the juvenile hormone titer in females of *Leptinotarsa migratoria* stopped vitellogenin production and, therefore, lead to sterility in females.

Results of this study have validated the use of *A. indica* as an anthelmintic by the local farming communities. The comparative anthelmintic activity of different fractions of *A. indica* points to the presence of active anthelmintic principles in ethyl acetate fractions. Therefore, future studies on drug development (etc.) may be focused on the ethyl acetate fractions of *A. indica*.

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