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RESEARCH ARTICLE

Anthelmintic Activity of a Herbal Formulation Against Gastrointestinal Nematodes of Sheep

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ABSTRACT

This study was carried out to evaluate the anthelmintic activity of a herbal formulation (HF) based on aqueous extracts of leaves of *Azadirachta indica* and *Nicotiana tabacum,* flowers of *Calotropis procera* and seeds of *Trachyspermum ammi*. *In vitro,* eggs and adult *Haemonchus contortus* were exposed to different concentrations of HF following the standard procedures of egg hatch test (EHT; 50 to 0.024414 mg ml^{-1}) and adult motility assay (AMA; 200-0.1953125mg ml^{-1}), respectively. The reference drugs used in the study were oxfendazole (0.0056704 to 0.0000027 mg ml^{-1}) and levamisole (1.50 mg ml^{-1}) for EHT and AMA, respectively. *In vivo,* pre and post-treatment (4 mg, 2 mg and 500 µg kg^{-1} body weight) fecal egg counts were determined following standard fecal egg count reduction test in sheep naturally parasitized with mixed species of gastrointestinal nematodes. In EHT, LC_{50} values of HF and oxfendazole (reference drug) were 275.1 and 0.016 µg ml^{-1}, respectively. In AMA, 100% mortality of *H. contortus* was observed 6 hr post-exposure to 3.125-200 mg ml^{-1} concentrations of HF and 2 hr post-exposure to levamisole. *In vivo,* maximum (96.2%) fecal egg count (EPG) reduction was recorded in sheep treated with HF @ 4 mg kg^{-1} body weight; whereas, 89.3% reduction in EPG was recorded in sheep treated with levamisole @ 7.5 mg kg^{-1} body weight. A graded dose response was noted in all the tests used in the present study to evaluate the anthelmintic activity of HF. Therefore, HF seems to be promising as an anthelmintic for animals. Large scale trials on efficacy and safety, however, are recommended before the HF is considered for commercialization in crude form.

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INTRODUCTION

Gastrointestinal nematodes (GIN) impair animal productivity through reduction in voluntary food intake and/or inefficient use of nutrients. Disturbance in protein metabolism and reduced absorption and/or retention of minerals are significant during parasite infection (Waller, 1987). The control of GINs predominantly depends upon chemotherapy despite advancements in genetical, immunological and biotechnological methods (Lloyd-Evans, 1991). Development of drug resistance in the parasites, effect of drug residues on human (Bowman *et al*., 1999) and high costs of the synthetic drugs, however, have led to attention of the workers to find the alternatives to the existing chemicals used for the treatment and/or control of parasites (Al-Shaibani *et al.,* 2009; Bachaya *et al.,* 2009; Deeba *et al.,* 2009; Sindhu *et al.,* 2010). Plants and/or their products have been used for treatment of different diseases for centuries. Anti-parasitic efficacy of different plants using standard parasitological procedures has been reported earlier (Dolan *et al*., 2007). In the present study, a combination of the aqueous extracts of leaves of *Azadirachta* (*A.*) *indica* (locally known as Neem) and *Nicotiana* (*N.*) *tabacum* (locally known as Tambaku), flowers of *Calotropis* (*C.*) *procera* (locally known as Aak) and seeds of *Trachyspermum* (*T.*) *ammi* (locally known as Ajwain) have been tested for their anthelmintic activity.

MATERIALS AND METHODS

Extraction of plant materials: Leaves of *A. indica* and *N. tabacum,* flowers of *C. procera* and seeds of *T. ammi* were obtained from field and/or local market (Faisalabad-

Pakistan). The plant materials were identified and authenticated by a botanist in the Department of Botany, University of Agriculture, Faisalabad (UAF), Pakistan, and dried under shade. Extraction was carried out in the Department of Parasitology, UAF. Five kilograms of each plant material was soaked together in 50 liters of distilled water at room temperature in large sized buckets covered with a lid. The suspension was shaken vigorously every 72 hrs. After 30 days, the suspension was concentrated at 25-30°C till the volume reduced to 20 liters. Then, it was further reduced to the weight of 10 kg in a vacuum oven. The total w/w yield of extract was 20%. The extract was stored at 4°C, and used for different biological assays after dilution with distilled deionized water as desired.

***In vitro* anthelmintic activity**

Egg hatch test (EHT): The EHT was performed following Coles *et al*. (1992). Adult *H. contortus* worms were obtained from the abomasal contents of slaughtered sheep. The female worms were separated and crushed in mortar and pestle to liberate the eggs. The concentration of eggs were estimated in 50 µl samples and adjusted to 100–150 eggs ml^{-1}.

Test procedure: One ml suspension containing approximately 100-150 freshly collected *H. contortus* eggs was added to each well of a 24-well flat-bottomed microtitration plate. The eggs were exposed to different concentrations (50 to 0.024414 mg ml^{-1} in distilled deionized water) of herbal formulation (HF) except in the positive and negative control wells having egg suspension with oxfendazole (0.0056704 to 0.0000027 mg ml^{-1}) and distilled deionized water, respectively. The microtitration plates were incubated for 48 hrs at room temperature followed by addition of Lugol's iodine solution in the wells. Un-hatched eggs and first-stage larvae (L_1) were counted in each well in all the plates. The experiment was carried out in triplicate for both control and extract. The LC_{50} of herbal extract and oxfendazole were calculated following Finney (1971).

Adult motility assay: Mature live *H. contortus* (females) was obtained from abomasums of sheep freshly slaughtered in the local abattoir. After washing, the collected worms (n=10) were suspended in phosphate buffer saline (PBS). Afterward, they were exposed to each of the following treatments in separate petri-dishes, in triplicate, at room temperature (25-30°C). PBS was added in all the petri-dishes. The negative control dishes contained more PBS so that all the dishes possessed uniformed volume.

1. The HF @ 200, 100, 50, 25, 12.5, 6.25, 3.125, 1.5625, 0.78125, 0.390625 and 0.1953125 mg ml^{-1}
2. Levamisole HCl @ 1.50 mg ml^{-1}
3. PBS (control)

The worms were observed after every two hours up to 8 hrs. Finally, the treated and negative control worms were kept in the lukewarm fresh PBS for 5 min to assess the restoration of motility and immotile worms were supposed as dead. The number of live and dead worms was recorded in all the Petri-dishes.

***In vivo* anthelmintic activity**

Fecal egg count reduction test: For each dose of HF (4 mg, 2 mg and 500 µg kg^{-1} body weights) of extract, and two control groups; 25, 4–8-months-old sheep (either sex) naturally infected with mixed gastrointestinal nematodes species were selected from the private farms around district Jhang. The animals having ≥ 750 eggs per gram of feces (EPG) were included in the study. The experimental animals were stall fed on a uniform ration for all the groups during the days of experimentation. Water was offered *ad libitum*.

Test Procedure: Before starting treatment, EPG of each animal was calculated from the fecal samples obtained directly from rectum, at least three times within the period of three days. Coprocultures were done for identification of the nematode species composition following MAFF (1979). Experimental sheep were distributed into five groups (A-E) through complete randomized design under consideration of their live weight and EPG on day 0. Group A was negative control while group B served as a positive control. Levamisole HCl @ 7.5 mg kg^{-1} (ICI Pakistan Limited, Animal Health Division) was drenched once to positive control group.

Groups C to E were drenched with single dose of the HF @ 4 mg, 2 mg and 500 µg kg^{-1} body weights. Fecal examination of each animal was carried out by the salt floatation technique (MAFF, 1979) in the morning on day 0, 3, 6, 9, 12 and 15 days post-treatment and EPG were calculated by the McMaster method (Soulsby, 1982). The following formula was used to calculate the percent egg count reduction (ECR):

$$\text{ECR (\%)} = \frac{\text{Pre-treatment egg count per gram–Post-treatment egg count per gram}}{\text{Pre-treatment egg count per gram}} \times 100$$

Statistical analysis: LC_{50} of HF and oxfendazole were calculated following Finney (1971) and probit was analyzed by Hubert and Kerboeuf (1992) method. In adult motility assay, the comparison between means of dead worms was assessed through DMR Test. For FECRT, the results were expressed as eggs per gram (Mean±SEM) of feces and means were compared by using DMR Test (SAS, 1998).

RESULTS

***In vitro* anthelmintic activity**

Egg hatch test: Exposure of *H. contortus* eggs to HF resulted in a dose dependent inhibition of egg hatching. There was, however, a large difference between the LC_{50} of HF (275.1 µg ml^{-1}; Table 1) and that of oxfendazole (0.016 µg ml^{-1}; Table 2). The 95% confidence intervals were 231.3 to 327.1, and 0.013 to 0.020; and the fitness of the model based on chi square analysis were P 0.263 and P 0.192 for HF and oxfendazole, respectively.

Adult motility assay: HF exhibited anthelmintic effects in a dose dependent manner and all the worms were found dead at 6 hr post-exposure at 3.125 mg ml^{-1} and above (Table 3). Mortality of worms was comparable to that

Table 1: LC_{50} and percentage of eggs hatching at various concentrations of the herbal formulation

Dose (mg ml^{-1})	Dose(µg ml^{-1})	hatch %	Log dose	Probit	Regression
0.024414	24.41406	70	1.38764	5.524	5.667744
0.048828	48.82813	65	1.68867	5.385	5.37073
0.097656	97.65625	63	1.9897	5.33	5.073716
0.195313	195.3125	45	2.29073	4.87	4.776702
0.390625	390.625	33	2.59176	4.56	4.479688
0.78125	781.25	18	2.89279	4.085	4.182674
1.5625	1562.5	9	3.19382	3.659	3.88566
3.125	3125	7	3.49485	3.524	3.588646
6.25	6250	6	3.79588	3.445	3.291632
12.5	12500	1	4.09691	2.676	2.994618
25	25000	1	4.39794	2.676	2.697604
50	50000	1	4.69897	2.676	2.40059

LC_{50} = 275.1 µg ml^{-1}

Table 2: LC_{50} and percentage of eggs hatching at various concentrations of oxfendazole

Dose (mg ml^{-1})	Dose(µg ml^{-1})	hatch %	Log dose	Probit	Regression
0.0000027	0.0027	98	2.56864	7.054	7.347628
0.0000055	0.0055	97	2.25964	6.881	7.125581
0.000011	0.011	96	1.95861	6.751	6.90926
0.0000221	0.0221	95	1.65561	6.645	6.691524
0.0000443	0.0443	96	1.3536	6.751	6.474498
0.0000886	0.0886	95	1.05257	6.645	6.258178
0.0001772	0.1772	95	0.75154	6.645	6.041857
0.0003544	0.3544	80	0.45051	5.842	5.825536
0.0007088	0.7088	75	0.14948	5.674	5.609215
0.0014176	1.4176	69	0.151554	5.496	5.392895
0.0028352	2.8352	47	0.452584	4.925	5.176574
0.0056704	5.6704	31	0.753614	4.504	4.960253

LC_{50} = 0.016 µgml^{-1}

Table 3: *In vitro* effect of the herbal formulation(HF) on survival of *Haemonchus contortus* of sheep in comparison with levamisole

Treatments	Mean (±SD) number of dead worms at different hrs post-exposure[1]				
	0 hr	2 hrs	4 hrs	6 hrs	8 hrs
Levamisole @ 1.50 mg ml^{-1}	0.0±0.00 ^{k}	10±0.00^{a}	10±0.00 ^{a}	10±0.00 ^{a}	10±0.00 ^{a}
PBS	0.0±0.00 ^{k}	0.0±0.00 ^{k}	0.0±0.00 ^{k}	0.0±0.00 ^{k}	0.0±0.00 ^{k}
HF (mg ml^{-1})					
0.195	0.0±0.00 ^{k}	0.0±0.00 ^{k}	0.0±0.00 ^{k}	0.0±0.00 ^{k}	2.66±1.52 ^{i}
0.390	0.0±0.00 ^{k}	0.0±0.00 ^{k}	0.0±0.00 ^{k}	1.67±1.52 ^{j}	2.33±1.52 ^{i}
0.781	0.0±0.00 ^{k}	0.0±0.00 ^{k}	4.33±0.57 ^{h}	5.66±1.15 ^{g}	7.33±1.52 ^{de}
1.562	0.0±0.00 ^{k}	5.67±1.15^{g}	6.33±1.15 ^{ef}	9.33±0.57 ^{ab}	10±0.00 ^{a}
3.125	0.0±0.00 ^{k}	6.00±1.00^{f}	7.33±0.57 ^{de}	10±0.00 ^{a}	10±0.00 ^{a}
6.25	0.0±0.00 ^{k}	7.33±0.57 ^{de}	8.00±1.00 ^{d}	10±0.00 ^{a}	10±0.00 ^{a}
12.5	0.0±0.00 ^{k}	7.33±0.57^{de}	8.00±1.00 ^{d}	10±0.00 ^{a}	10±0.00 ^{a}
25	0.0±0.00 ^{k}	8.33±0.57 ^{cd}	9.00±0.00 ^{b}	10±0.00 ^{a}	10±0.00 ^{a}
50	0.0±0.00 ^{k}	8.33±0.57^{cd}	9.33±0.57^{ab}	10±0.00 ^{a}	10±0.00 ^{a}
100	0.0±0.00 ^{k}	8.67±0.57^{bc}	10±0.00 ^{a}	10±0.00 ^{a}	10±0.00 ^{a}
200	0.0±0.00 ^{k}	9.0±1.00^{b}	10±0.00 ^{a}	10±0.00 ^{a}	10±0.00 ^{a}

[1]Each treatment group had three replicates each having 10 worms; the values with same superscript in a row do not differ significantly at P≥0.05.

Table 4: *In vivo* anthelmintic activity of the herbal formulation (HF) based reduction in eggs per gram of feces (Mean+SE) in sheep naturally parasitized with gastrointestinal nematodes

Days PT	Doses of HF			Control groups	
	500 µgkg^{-1} body weight	2 gkg^{-1} body weight	4 gkg^{-1} body weight	Levamisole	Negative Control
0	2526.7±200 ^{a}	2214±203 ^{a}	5053.3±209 ^{a}	2487.6±219 ^{a}	5335.5±198 ^{a}
3	1972.6±336.5^{b} (21.9)	1370.9±404.9^{b} (38.1)	2285±465.9^{b} (54.8)	862.8±382.9^{b} (65.3)	5320.3±101.1 ^{a} (0.3)
6	1743.3±307.1^{c} (31.0)	591.6±169.6^{c} (73.3)	1099.7±261.7^{c} (78.3)	606.4±306.6^{c} (75.6)	5298.7±120.2 ^{a} (0.7)
9	1736.7±308.3^{c} (31.3)	338.3±70.4^{d} (84.7)	448.5±74.4^{d} (91.1)	453.9±288.0^{d} (81.8)	5307.1±120.5 ^{a} (0.5)
12	1780±306.7^{c} (29.6)	254±47.8^{e} (88.5)	317.5±61.8^{e} (93.7)	321.9±279.0^{e} (87.1)	5290.4±166.8 ^{a} (0.8)
15	1859.5±312.6^{d} (26.4)	186.5±18.0^{f} (91.6)	190.9±39.0^{f} (96.2)	265.9±279.0^{f} (89.3)	5300.9±174.8 ^{a} (0.6)

Each group had five sheep; PT= Post-treatment; Values with different superscripts in a column differ significantly (P≥0.05). Figures in parenthesis indicate percentage.

with levamisole (reference drug) at 200 mg ml^{-1} at 2 hr post-exposure. There was no mortality of worms in PBS till 8 hrs.

***In vivo* anthelmintic activity:** The experimental sheep were found to have mixed infection with *H. contortus, Chabertia ovina, Trichostrongylus spp., Telodorsagia spp.* and *Oesophagostomum columbianum.* HF exhibited anthelmintic activity in a time and dose-dependent manner. The maximum reduction (96.2%) in eggs per gram of feces (EPG) was recorded on day 15 post-treatment in sheep treated @ 4 g kg^{-1} body weight (Table 4). The reduction in EPG (89.3%) with levamisole was comparable to that with the HF @ 4 g kg^{-1} body weight.

DISCUSSION

Results revealed anthelmintic efficacy of the HF in all the tests used in this study. The plants used in the HF have been reported for their anthelmintic activities individually

in numerous studies. For example, crude methanolic extract of *A. indica* leaves have been reported for their ovicidal (LC50=371.53 µg ml^{-1}), larvicidal (LC50=199.53 µg ml^{-1}) and wormicidal (>500 µg ml^{-1}) effects (Bachaya, 2007). Iqbal *et al.* (2010) have demonstrated mild anthelmintic activity of crude powder and methanolic extract of *A. indica* seeds as evident from 29.3 and 40.2% reduction in EPG, respectively in sheep naturally parasitized with mixed species of gastrointestinal nematodes (GINs). In contrast; however, Worku *et al*. (2009) could not demonstrate anthelmintic effects of *A. indica* leaves. Iqbal *et al.* (2006) have reported *in vitro* and *in vivo* anthelmintic activity of crude aqueous and methanol extracts of *N. tabacum* leaves. Aqueous extract was more effective in *in vitro* adult motility assay; whereas, methanolic extract caused higher reduction (73.6%) in EPG compared with aqueous extract (49.4%) in sheep naturally parasitized with GINs. Bachaya (2007) has also demonstrated ovicidal (LC_{50}=13.96 µg ml^{-1}) and larvicidal (LC_{50}=478.63 µg ml^{-1}) effects of methanolic extract of *N. tabacum* leaves against *H. contortus*. Crude powder, and/or aqueous and methanolic extracts of *C. procera* flowers have been reported for their *in vitro* and *in vivo* anthelmintic activity (Iqbal *et al*., 2005). *In vitro,* methanolic extract was more effective; whereas, aqueous extract (88.4%) and crude powder (77.8%) caused higher reduction in EPG compared with methanolic extract (20.9%) in sheep naturally parasitized with GINs. Shivkar and Kumar (2003) have also reported *in vitro* inhibition of motility of adult earthworms exposed to aqueous extract of dried latex of *C. procera.* Seeds of *T. ammi* were validated for their anthelmintic activity by Lateef *et al.* (2006). Crude powder caused higher (78.1%) reduction in EPG compared with aqueous extract (53.3%) in sheep naturally parasitized with GINs. Jabbar *et al.* (2006) have also demonstrated ovicidal effects of aqueous (0.1698 mg ml^{-1}) and methanolic (0.1898 mg ml^{-1}) extracts of *T. ammi* seeds against *H. contortus* eggs.

HF tested in this study proved to have ovicidal and wormicidal effects *in vitro* against *H. contortus*, and caused reduction in EPG better (96.2%) than that with levamiosle, the reference drug (89.3%) in sheep naturally parasitized with GINs. These findings have suggested that (i) HF seems effective against populations of nematodes resistant to levamisole, (ii) combination of aqueous extracts of different plants in the HF exhibited higher anthelmintic effects compared with their individual efficacy as described above, (iii) HF is based on plants commonly available on the doorstep of the rural communities and (iv) HF is cheap compared with the synthetic anthelmintics & can easily be prepared by simple extraction procedures. Better efficacy of HF than that of the individual plants point to some sort of synergistic effects among the phytochemicals of the plants used.

Phytochemical studies of the ingredient plants show that latex and flowers of *C. procera* accounted for phenolic compounds/tannins, carbohydrates, alkaloids, glycosides, amino acids and proteins, flavonoids, saponins, acidic compounds and sterols (Sharma and Sharma, 1999). The active ingredients responsible for anthelmintic properties are not elucidated so far. However, Kumar and Shivkar (2004) recorded spasmogenic effect of latex on muscles of rats (gastrointestinal). Likewise, the aqueous extract of *C. procera* exhibited spasmolytic effect on muscle chain (smooth) of trachea in guinea-pig (Iwalewa *et al*., 2005). *A. indica* contained triterpenol derivatives such as nimbin, azadirachtin, salannin, diacetyl-nimbin (Verma *et al.,* 1995) and its anthelmintic properties are attributed to azadirachtin (Sharma *et al.,* 2003). *N. tabacum* possesses nicotine, a ganglion stimulant, which may be responsible for its anthelmintic properties through the activation of neuromuscular junctions leading to spastic paralysis, death and expulsion of worms from the body of host (Neal, 2002). Major chemical constituent of *T. ammi* is Thymol (Khajeh *et al*., 2004). Thymol may have exerted anthelmintic actions (Sánchez *et al*., 2004) by (i) increasing surface curvature and polarity of the membranes at the level of packing densities of natural membranes, (b) activating and stimulating the binding of GABAA-R to FNZ at the extent of concentration more than critical micellar concentration, and (c) effecting the binding (allosteric) sites of GABA leading to disturbances in the coupling of GABA with FNZ.

In conclusion, HF tested in this study is conveniently available, economical and effective against GINs of sheep. Standardization of procedures for its preparation, doses, and large scale trials are recommended before the tested HF is considered for commercialization.

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