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RESEARCH ARTICLE

Antinematicidal Activity of *Nicotiana tabacum* L. Leaf Extracts to Control Benzimidazole-Resistant *Haemonchus contortus* in Sheep

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ABSTRACT

This study was carried out to assess the efficacy of *Nicotiana* (*N.*) *tabacum* leaves to control benzimidazole-resistant *Haemonchus* (*H.*) *contortus* in sheep. Fecal egg count reduction test (FECRT) and egg hatch assay (EHA) revealed catastrophic levels of resistance in *H. contortus* of sheep against oxfendazole, a member of benzimidazole group of antinematicidals. Antinematicidal efficacy of *N. tabacum* leaves was evaluated employing EHA, adult motility test (AMT) and FECRT. There was a dose and time dependent antinematicidal activity of crude aqueous methanol extract (CAME) of *N. tabacum* leaves with estimated LC_{50} values of 0.566 and 1.91 mg ml^{-1} in EHA and AMT, respectively. There was, however, no significant-difference ($P>0.05$) in fecal egg count reduction (87.5 vs 88.6%) in sheep at low (2 g kg^{-1} BW) and high (4 g kg^{-1} BW) doses of CAME of *N. tabacum* leaves. Administration of *N. tabacum* leaves at low dose (2 g kg^{-1} BW) did not exhibit untoward effects in animals; therefore, may be used in sheep harboring antinematicidal- resistant populations of *H. contortus*.

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INTRODUCTION

Ovine and caprine are prone to infection with *Haemonchus (H.) contortus* throughout the world inflicting heavy economic losses in small ruminant industry due to high mortality in addition to reduction in productivity (Lashari and Tasawar, 2011). The control strategies of this trichostrongylid parasite rely mainly on synthetic chemotherapeutics. Unfortunately, the recurrent use of synthetic dewormers has led to emergence of antinematicidal resistance among *H. contortus* populations in most countries of the world including Pakistan (Saddiqi *et al*., 2006). The above reasons have encouraged workers in the field of parasitology to investigate indigenous medicinal plants as effective substitutes to fight parasitism in livestock (Sindhu *et al*., 2010; Badar *et al*., 2011). Earlier, some studies have been conducted on evaluation of plants for their antiparasitic activity (Iqbal *et al*., 2006; Sindhu *et al*., 2012). These studies, however, have not focused on the efficacy of botanicals against known drug-resistant population of parasites. *Nicotiana* (*N.*) *tabacum* leaves, commonly used as an anthelmintic in ethnoveterinary medicine in Pakistan, have also been validated for their anthelmintic activity (Iqbal *et al*., 2006) but not against the known drug-resistant *H. contortus*. The current study was, therefore, conducted to evaluate the efficacy of *N. tabacum* leaves against benzimidazole-resistant *H. contortus.*

MATERIALS AND METHODS

Study site: This study was conducted at Angora Goat Farm, Rakh Khairewala- Layyah District, Pakistan. Farm is situated in the hot semi-arid area and the climate is sub-tropical in the region. Maximum and minimum temperature has been recorded as 50°C and 4°C in summer and winter, respectively. The annual rainfall ranges from 150-200 mm from July to early September and 50 mm from November to February. The humidity ranges from 35 to 60% in different parts of the year. The sheep flocks maintained on the farm are of indigenous Pak Karakul breed. Sheep graze on natural pastures during day time and, penned and fed on dry roughages from evening to morning. Green fodders are fed rarely and no concentrate is offered to animals. Benzimidazoles (oxfendazole and albendazole), imidazothiazoles

(levamisole HCL) and macrocyclic lactones (ivermectin) were the most commonly used anthelmintics on the farm.

Selection of animals for study: Sheep (n=60) with meeting the following criteria were selected for the study:
Age: 3-6 months (Coles *et al*., 1992)
Deworming history: not have been dewormed for the last 8-12 weeks (Coles *et al*., 1992)
Anemic: stage 3, 4 or 5 [FAMACHA Anemia Guide Chart (Macedo *et al*., 2010)]
Eggs per gram of feces (EPG): minimum 150 eggs (Coles *et al*., 1992)
H. contortus infection: >90% (Bowman *et al*., 2003)
The animals selected for the study were separated from the herd being maintained on the farm, randomly divided and tagged into the following four groups:
Group 1: Oxfendazole resistance detection group (n=15)
Group 2: *N. tabacum* low dose (2 g kg^{-1} BW) treated group (n=15)
Group 3: *N. tabacum* high dose (4 g kg^{-1} BW) treated group (n=15)
Group 4: Infected untreated group (n=15)
The experimental animals were housed separately and fed on rough fodder and did not receive any kind of other treatment during the period of trial.

Procedures

Fecal examination: Qualitative and quantitative fecal examinations of all the animals were carried out during the selection process. Fecal egg count reduction test (FECRT) was employed for detection of antinematicidal resistance and evaluation of *N. tabacum* leaves against antinematicidal-resistant *H. contortus* following standard parasitological procedures (Soulsby, 1982; Coles *et al.*, 1992).

Coproculture: Coprocultures were also performed to assess the contribution of different species of nematodes in overall natural helminth infections following MAFF (1986) during the selection process. Fecal samples from each group of animals were pooled and cultured in glass dishes. The cultures were incubated for seven days at 27±1°C. After this period, the larvae (L3) were recovered employing Baermann apparatus. Lugol's iodine was added to the cultures and 100 larvae were counted and identified according to MAFF (1986).

Antinematicidal resistance studies

FECRT: Oxfendazole [2.265%; Epla Lab. (Pvt.) Ltd.] was purchased from market and tested for its purity by HPLC analysis in Central Hi-Tech Laboratory, University of Agriculture, Faisalabad (UAF), Pakistan. The animals (n=15) in group 1 were treated with oxfendazole at the recommended dose (5 mg kg^{-1} BW); whereas, group 4 served as infected untreated control. Fecal examinations and coprocultures of the animals were carried out at day 0 (pre-treatment) and day 14 (post-treatment) as described above. Record of pre- and post oxfendazole treatment EPG and composition of nematode infections were kept. Faecal egg count reduction percentage (FECR %) was calculated by the following formula:
FECR% = [1-(mean EPG treatment/mean EPG control)] x 100

RESO spreadsheet (CSIRO Animal Health Research Laboratory, Private Bag 1, Parkville, Vic. 3052, Australia) was used to compute the FECR data including arithmetic mean, variance of counts, FECR% and 95% confidence interval. According to Coles *et al.* (1992), resistance is developed if (i) the FECR% is less than 95% (ii) the lower limit of 95% confidence interval is less than 90% (iii) If just one of the two norms is met resistance is suspected. Any negative values calculated from faecal egg reduction percentage and lower limit of confidence interval were considered equal to zero, meaning thereby that the resistance is highly prevalent and at the catastrophic level as suggested by Gill (1996).

EHA was performed following the standardized protocol that was accepted by W.A.A.V.P. (Coles *et al*., 1992) to detect resistance against oxfendazole. About 10 g of faeces was taken from the pooled samples and added to a 100 ml screw-top plastic bottle containing 10-12 glass beads and 85 ml tap water. The sample bottle was capped, shaken for one minute to disperse the faecal material. The sample was placed in ice and transported to the Chemotherapy laboratory, Department of Parasitology, UAF, and stored at 20°C. Faecal samples were homogenized with a stirrer and filtrated through a 100 mesh (0.15 mm aperture). The filtrate was centrifuged using eight tubes for two minutes at 300x*g*, and supernatant was discarded. The tubes were stirred up to loosen the sediment, then saturated sodium chloride was added until a meniscus formed above the tube, a glass cover slip was put and centrifuged for 2 minutes at 130x*g*. Cover slips were removed and eggs were washed off into a conical glass centrifuge tube. The tubes were filled with water and centrifuged for 2 minutes at about 300x*g*. Water was sucked off and the eggs were resuspended in water and ultimately the number of eggs was estimated using the modified McMaster technique (Coles *et al*., 1992).

Oxfendazole (98.52% pure standard reference from Sanna Laboratories) was dissolved in 0.3% Dimethylsulfoxide (DMSO) and stock solution was prepared as 25 µg ml^{-1}. The stock solution was serially diluted (0.0122-25 µg ml^{-1}) in a 24 multiwell plate (Flow Laboratories). The control well received only 1 ml solvent (0.3% DMSO). One ml (150 eggs ml^{-1}) of egg suspension (anaerobically stored) was added to each well including control well. There were three replicates for each concentration and control. Plate was incubated at 27°C for 48 hours and 70% relative humidity. After incubation, two drops of Lugol's iodine was added. At least 100 of the unhatched eggs (dead and embryonated) and hatched larvae were counted to calculate the hatching inhibition percentage (Coles *et al*., 1992).
The following formula was utilized for estimation of hatching inhibition (%):
Hatching inhibition (%) = P test/ P total ×100
P test: number of unhatched or embryonated eggs.
P total: number of unhatched or embryonated eggs + Larvae (L1)

LC_{50} values were calculated for the eggs by probit analysis. Eggs with an LC_{50} value in excess of 0.1 µg ml^{-1} were considered as an indication of antinematicidal (oxfendazole) resistance as proposed by Coles *et al*. (1992).

Efficacy of *Nicotiana tabacum* extracts against resistant *Haemonchus contortus*

Plant extraction: *N. tabacum* leaves were procured from the local market of Faisalabad-Pakistan, cleaned from contaminants (like fungi, etc.) and dried. The plant leaves were pulverized finely to a powder in an electric grinding machine and kept in cellophane bags. The ground plant materials were steeped in 70% aqueous methanol (the concentration adjusted by an alcohol meter) by cold maceration at room temperature and the mixture was stirred two times daily. After three days, the filtrate was collected through a piece of porous cloth and the plant materials re-steeped again in 70% aqueous methanol. The above described process was repeated thrice. The combined filtrates were concentrated in a rotary evaporator at 40°C under reduced pressure, and for more evaporation, vacuum- oven at 40°C was also used to prepare crude aqueous-methanol extracts (CAMEs) (Gilani *et al*., 2004). These extracts were stored at 4°C until used against the parasite employing *in vivo* and *in vitro* methods. The percentage yield of extract was calculated as under:

Ratio= A/B×100

A: weight yield after extraction (g)

B: dry matter weight (g)

FECRT: The animals (n=15) in group 3 and 4 were treated with CAME of *N. tabacum* leaves at low (2 g kg^{-1} BW) and high (4 g kg^{-1} BW) doses, respectively; whereas, group 4 served as infected untreated control. Fecal examinations and coprocultures of the animals were carried out at day 0 (pre-treatment) and day 14 (post-treatment) as described above. Record of pre- and post-CAME treatment EPG and composition of nematode infections were kept. FECR % was calculated by the following formula:

FECR% = [1-(mean EPG treatment/mean EPG control)] x 100

EHA was carried out to evaluate the inhibitory effects of different concentrations of the CAME on hatching of the parasite eggs. Virtually, the assay was conducted pursuant to the methodology described by Coles *et al*. (1992) with minor modifications by some researchers to be more suitable for assessment of plants (Macedo *et al*., 2010). One gram of CAME was dissolved in 10 ml of 70% acetone and this was considered as stock solution (100 mg ml^{-1}) which was serially diluted in a 24 multiwell plate. The egg samples were exposed to 12 concentrations (100-0.048 mg ml^{-1}) of the CAME. For positive control, 0.025 mg ml^{-1} of oxfendazole was prepared with 0.3% DMSO (solvent). The negative control well received only 1ml of 70% acetone. LC_{50} was calculated using probit analysis. The other steps were same as followed in studies on antinematicidal resistance.

Adult motility test (AMT) was employed with minor modifications to determine the impact of the plant extracts on the viability of live adult resistant *H. contortus* in sheep (Singh *et al*., 1985). The mature worms of either sex were collected from the abomasa of two experimental animals slaughtered at the end of the experiment. The recovered worms were washed and suspended in PBS. Acetone (70%) and PBS (50:50 v/v) were used to dissolve the CAME. The stock solution (100 mg ml^{-1}) was serially diluted (two-fold serial dilution) in PBS to prepare various concentrations (100-0.048 mg ml^{-1}) in a 24-well flat-bottomed titration plate. The positive control received 25 µg ml^{-1} of closantel dissolved in PBS, whilst the negative control well contained 1 ml of 70% acetone and 1 ml of PBS. The experiment was done at room temperature (25-30°C). Ten live worms were added to each well containing CAME and, positive and negative control. The worms were observed on 0, 2, 4, 6, 8, 10 and 24 hours for their motility, mortality and paralysis. There were three replicates for each concentration.

Statistical analysis: The data obtained from different experiments were analyzed statistically using RESO Computer Programme for detection of resistance among the parasite populations after performing FECRT (Coles *et al*., 1992). Probit analysis was utilized for calculation of LC_{50} (Hubert and Kerboeuf, 1992). Completely randomized design (CRD) was applied for detection of resistance against oxfendazole through performing EHA and for evaluation of the ovicidal activity of *N. tabacum*. For analyzing the data procured from FECRT, Tukey's test was applied and F-values were calculated. To compare between low dose and high dose of the used plant, t- test was applied. Analysis of data collected from AMT, 3- factor (time, concentration, death) CRD was applied. Significant differences among various means were determined using Tukey's test at 5% level of significance.

RESULTS

Composition of natural nematode infections in experimental animals: The animals included in the experiment were predominantly infected with *H. contortus*; however, other nematode species were: *Chabertia* spp., *Trichostrongylus* spp., *Teladorsagia* spp., and *Oesophagostomum* spp. Coproculture revealed *H. contortus* as the major contributor to the EPG (>90%) of the experimental groups of animals (Table 1).

Resistance studies

Coproculture: As evident from the results of pre-treatment coproculture of pooled faecal samples of all animals, the oxfendazole and control groups had 96 and 93% *H. contortus* infection respectively, whilst infection with the other species of nematodes was 4 and 7% for oxfendazole and control groups, respectively (Table 1). Post-treatment (day 14), the oxfendazole and control groups had 97 and 95% *H. contortus* infection respectively, whilst infection with the other species of nematodes was 3 and 5% for oxfendazole and control groups, respectively.

FECRT: Mean EPG reduction and FECR% on day 14 post-treatment with oxfendazole based on calculations using RESO programme were 1543±413.27 SE (control mean EPG=987±364.26 SE) and -56 respectively. The calculated lower confidence interval 95% was -311. The data had indicated that the resistance was at catastrophic level in the farm.

EHA: The LC_{50} of oxfendazole was found to be 1.86 µg ml^{-1} (range 1.45-2.45), which was in excess to 0.1 µg ml^{-1} suggesting development of resistance against *H. contortus* (Coles *et al*., 1992). Correlation between effects of different concentrations of oxfendazole and hatching inhibition (%) through performing EHA is presented in Fig. 1.

Table 1: Pre-treatment composition of nematode infection in the animals selected for the study based on pooled faecal samples

Groups	*Haemonchus contortus* L3 (%)	L3 (%) of other nematodes
Oxfendazole	96	4
Nicotiana tabacum (L.D)	97	3
Nicotiana tabacum (H.D)	95	5
Control	93	7

L.D= Low dose (2 g kg^{-1} BW); H.D= High dose (4 g kg^{-1} BW).

Table 2: Mean±SE EPG reduction and mean FECR% in animals on day 10-14 post-treatment with CAME of *Nicotiana tabacum* plus comparison between effects of low and high doses of the plant extracts on the mean of egg reduction

Tests	Low dose	High dose	t-value	Probability
Mean EPG reduction	123.33±77.90	113.33±66.09	0.10^{NS*}	0.923
Mean FECR%	87.5	88.6	-	-
Control	Mean EPG= 987±364.26			

NS* = Non-significant (P>0.05).

Table 3: Calculated LC_{50} value for adulticidal activity of *Nicotiana tabacum* after performing adult motility test

Hours	LC_{50} (mg ml^{-1})	95% CI	
		Lower	Upper
0	-	-	-
2	32.68	19.24	66.97
4	8.24	5.09	14.55
6	1.91	0.83	1.70
8	0.47	0.31	0.68
10	0.20	0.12	0.28
24	-	-	-

CI = Confidence interval; 0 hr = All the worms were alive; 24 hr = All the worms were dead

Antinematicidal activity of *N. tabacum* leaves against resistant *H. contortus*: The yield of CAME from *N. tabacum* was 1.95%. Post-treatment coproculture of pooled faecal samples of all the animals included in low and high dose groups of *N. tabacum* has revealed very poor recovery of *H. contortus* and other nematode larvae (L3). In the control group, the percentage of *H. contortus* L3 was 95%, whilst recovered L3 for other nematodes was 5%.

FECRT: The results of antinematicidal activity of *N. tabacum* CAME (low and high doses) against oxfendazole-resistant *H. contortus* populations in the experimental animals naturally infected with the predominant parasite (>90%) in addition to the comparison between effects of low and high doses of the plant extracts on the mean of egg reduction is statistically analyzed in Table 2. The calculated t-value of egg reduction mean was less than table t-value which signifies non-significant difference between low and high doses and both doses were similarly effective.

As evident from the results of coproculture after treatment, the two groups exposed to effective *N. tabacum* extracts (FECR% >80), very few larvae of *H. contortus* and larvae of other nematodes were recovered.

EHA: The procured data from analysis of variance (ANOVA) of EHA concerning ovicidal activity of different concentrations of *N. tabacum* CAME in addition to calculation of the mean±SE of hatching inhibition (%) confirmed different effects of different concentrations (dose-dependent ovicidal activity) which is shown in Fig. 2. The calculated mean square was 3159.03 which denotes highly significant (P>0.01). The estimated LC_{50} was 0.566 mg ml^{-1} (range 0.496-0.642).

AMT: According to the data obtained from the adulticidal activity of *N. tabacum* CAME using AMT and calculated F-value from ANOVA table, there was significant difference (P<0.01) between the three factors (time, concentration and mortality). The mean mortality, after exposure of live resistant *H. contortus* to different concentrations of the plant extracts, was determined every two hours. The data are expressed in Fig. 3. The LC_{50} values at different hours are calculated (Table 3).

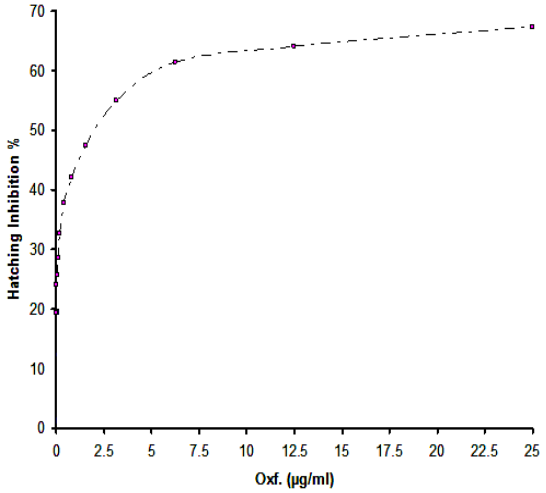

Fig. 1: Correlation between effects of different concentrations of oxfendazole and hatching inhibition (%) through performing egg hatch assay (Oxf= Oxfendazole).

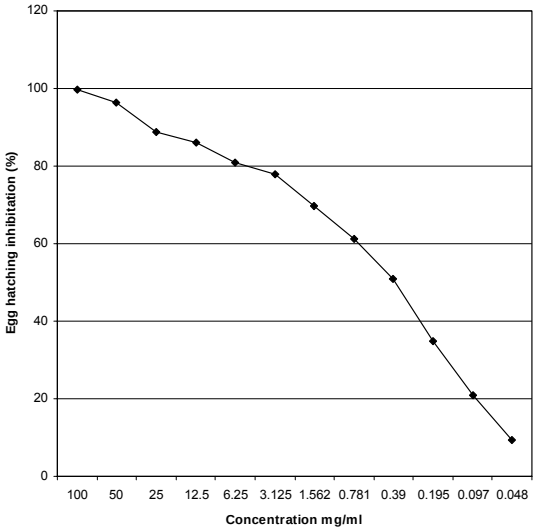

Fig. 2: Correlation between the effects of different concentrations of *Nicotiana tabacum* CAME and egg hatching inhibition (%).

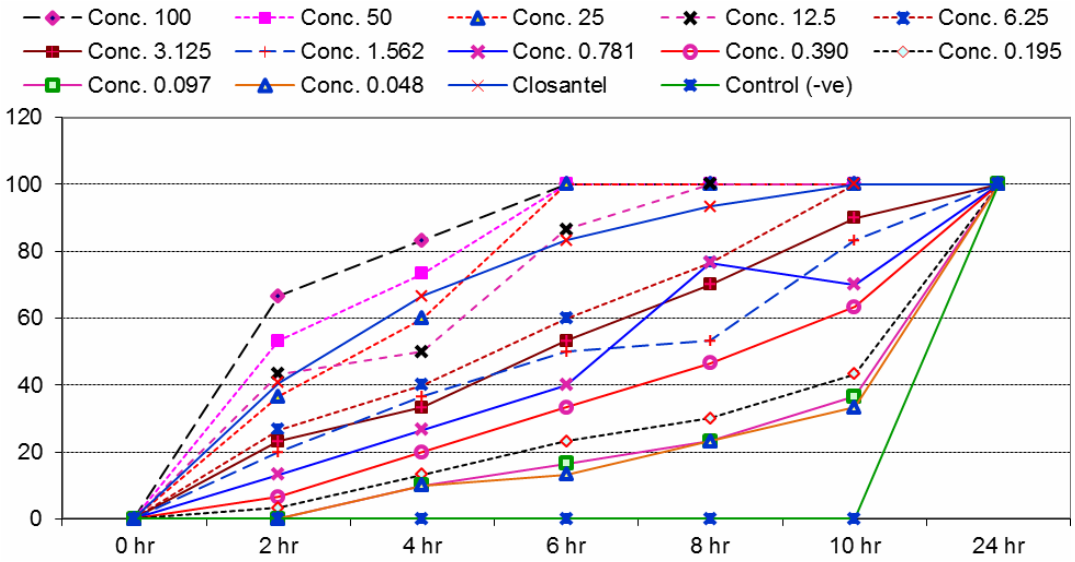

Fig. 3: Mortality (%) of resistant live adult *Haemonchus contortus* post-exposure to the CAME of *Nicotiana tabacum*. Conc. = Concentration of each CAME (mg ml^{-1}); Closantel concentration = (25µg ml^{-1}); Control (-ve) = 1 ml of 70% acetone plus 1 ml of PBS. All parasites are alive at 0 hr.

DISCUSSION

Antinematicidal resistance against oxfendazole (OXF) in GI nematodes (particularly *H. contortus*) of sheep has been detected earlier on Angora Goat Farm for survey purposes only (Saddiqi, 2005). The present study was elaborated and extended to re-confirm the rampancy of resistance among *H. contortus* populations in sheep in addition to assessment of *N. tabacum* CAME for its potency to control the OXF-resistant parasite. As far as it could be ascertained that this is the first report on efficacy of a botanical against the antinematicidal-resistant *H. contortus*. The calculated FECR% was (-56), whilst the lower confidence interval was (-311) which indicate that the resistance was at the catastrophic level (Gill, 1996) in the farm because even triple dose (15 mg kg^{-1} BW) was ineffective to reduce the parasitic load in naturally parasitized sheep.

LC_{50} value of OXF (1.86 µg ml^{-1}) calculated by probit analysis after conducting EHA also confirmed evolution of resistance among the parasite populations. In the light of observation that LC_{50} value higher than 0.1 µg ml^{-1} which is an evidence of BZ resistance (Coles *et al*., 1992), it can be concluded that resistance level among *H. contortus* populations in sheep in Angora Goat Farm is above 25% and the parasite is extremely resistant to OXF. It is noteworthy to mention that the antinematicidal resistance could not be detected by these conventional parasitological assays if the resistance level is below 25% among the GI nematode populations (Martin *et al*., 1989). Undoubtedly, the main reason behind the development of resistance among *H. contortus* populations to all the BZs is occurrence of mutation (loss of drug binding) at ß-tubulin isotype 1 (drug target) as a result of frequent annual use of the drug which causes selection pressure on the parasite (Beech and Silvestre, 2010). Coles *et al*. (1995) have reported evolution of antinematicidal resistance even when merely two to three drenches were administered annually. This kind of application of treatment and development of resistance were observed in the current study due to using BZ five times annually as average in Angora goat Farm. On the other hand, other contributing factors for development of resistance such as poor quality of dewormers, indiscriminate use of antinematicidal drugs (Afaq, 2003), non-rotation of anthelmintics, absence of any integrated strategic or tactical programme, unplanned grazing management and malnutrition should not be excluded on Angora Goat Farm (Local Veterinarian File).

CAME of *N. tabacum* leaves exhibited antinematicidal activity against OXF-resistant *H. contortus* in sheep in all the tests [FECRT, EHA and adult motility test (AMT)] employed in the study. Leaves of *N. tabacum* contain nicotine (comprises 90% of the total alkaloidal content of *N. tabacum*), a main ganglion inducer- alkaloid, which is possibly responsible for the antinematicidal activity of this plant extract. FECR% was statistically similar (P>0.05) in the animals treated at low (87.5%) and high (88.6%) doses of CAME. The LC_{50} of CAME in EHA was recorded as 0.566 µg ml^{-1} (range 0.496-0.642 µg ml^{-1}). In AMT, all worms were found dead at 6 hours post-exposure to 25 mg ml^{-1} of CAME. With decreasing concentration, mortality of the worms was delayed. CAME was even more effective than closantel, the reference drug at 25 µg ml^{-1} that killed all the worms 10 hours post-exposure. In negative control [50:50 v/v of 70% acetone and phosphate buffer saline (PBS) at PH 7.2], all worms were found dead after 24 hours. The LC_{50} of CAME in AMT was recorded as 32.68 mg ml^{-1} (range 19.24-66.97 mg ml^{-1}) two hours post-exposure, whilst the recorded LC_{50} value was 0.20 mg ml^{-1} (range 0.12-0.28) after 10 hours of exposure. Plants have been frequently reported to possess antimicrobial (Jahan *et al*., 2011; Oly *et al*., 2011; Lasalita-Zapico *et al*., 2012; Toroğlu *et al*., 2012), insecticidal (Yildirim *et al*., 2012) and antiparasitic (Waller *et al*., 2001) properties. The broad spectrum of the activities of plants is attributed to the diversity of chemicals present in plants like phenolics and polyphenols (simple phenols, phenolic acids, quinines, flavones, flavonoids, flavonols, tannins, coumarins, etc.),

terpenoids and essential oils, alkaloids, lectins and polypeptides, mixtures, other compounds, etc. Validation of the plants for their use in different ailments adds to their value and justifies especially in the rural areas, where diseases like parasitism (Farooq *et al.,* 2012) have huge negative impact on animal productivity due to inaccessibility of farmers to modern animal health care facilities.

Regarding variations in potency of this plant in the light of some studies, previous workers have recorded superior antinematicidal activity as compared with the results of the present study (Iqbal *et al.,* 2006). This may primarily be attributed to the differences among target worm populations, resistant status of the nematodes, type of extract used and source of the plant material. Other involving factors like biological activity of plants which depends on the source of plant, harvesting season (Hammond *et al*., 1997), mode of preparation of the dosage, variation within species of plant, storage technique and drying process (Croom, 1983) should be considered as well. In accordance to the recommendations of W.A.A.V.P. (Wood *et al*., 1995), an anthelmintic with FECR percentage 98 is considered highly effective, FECR percentage 80 and above is effective, whilst FECR percentage less than 80 is not recommended for use. So, in the light of the current study and W.A.A.V.P. recommendations, *N. tabacum* leaves may be classified as an effective antinematicidal.

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