



RESEARCH ARTICLE

Combination of *Nicotiana tabacum* and *Azadirachta indica*: A Novel Substitute to Control Levamisole and Ivermectin-Resistant *Haemonchus contortus* in Ovine

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ABSTRACT

The current study was aimed at exploring the activity of combined crude aqueous methanol extracts (CAMEs) of *Nicotiana (N.) tabacum* leaves and *Azadirachta (A.) indica* seed kernels to control levamisole (LEV) and ivermectin (IVM)-resistant *Haemonchus (H.) contortus* in sheep. The *in vivo* fecal egg count reduction test (FECRT) demonstrated rampancy of resistance among *H. contortus* populations against LEV and IVM. There was a significant difference ($P < 0.05$) between groups treated with the combined plant extracts compared to other groups treated with solitary *N. tabacum* and *A. indica*. The recorded FECR% at low ($1+1\text{g kg}^{-1}$ BW) and high ($2+2\text{g kg}^{-1}$ BW) doses was 52.71 and 94.59, respectively for the combined extracts, whilst for *N. tabacum* at low (2g kg^{-1} BW) and high (4g kg^{-1} BW) doses, the FECR% was 87.5 and 88.6, respectively. Moreover, the FECR% for *A. indica* was 45.62 and 85.14 at low (2g kg^{-1} BW) and high (4g kg^{-1} BW) doses, respectively. There was a dose and time dependent ovicidal and adulticidal activity of CAMEs with estimated LC_{50} values of 0.523, 0.566 and $1.169\ \mu\text{g ml}^{-1}$ for the combined extracts, solitary *N. tabacum* and *A. indica*, respectively in egg hatch assay (*in vitro* test). The estimated LC_{50} values in adult motility test (*in vitro* test) 10 hours post-exposure were 0.17, 0.20 and $0.80\ \text{mg ml}^{-1}$ for the combined extracts, sole *N. tabacum* and *A. indica*, respectively. Accordingly, the aforementioned combined extracts at high dose were more efficacious than solitary *N. tabacum* and *A. indica* and, thus, can be used to restrict rampancy of LEV and IVM-resistant *H. contortus* populations parasitized in sheep.

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INTRODUCTION

The fourth-stomach inhabitant, *Haemonchus (H.) contortus* is deemed a major contributor in reducing small ruminant productivity due to its deleterious pathologic effects on the parasitized animals, particularly in the tropical and subtropical zones in developing countries. Nematodiasis including haemonchosis in ovine and caprine is routinely treated with broad-spectrum synthetic anti-worm medications (benzimidazole, levamisole and ivermectin) at organized farms in most countries of the world (Taylor *et al.*, 2007) including Pakistan (Babar, 2005; Hamad *et al.*, 2013). Unhappily, the frequent annual

employment of the aforementioned antinematocidals has concluded to the evolution and rampancy of resistance among *H. contortus* populations (Neveu *et al.*, 2007; Farooq *et al.*, 2012). This, in turn, is a complicated dilemma that has emboldened parasitologists to promote alternative sources instead of synthetic chemotherapeutics (Waller, 2006). It is noteworthy to mention that the advocated substitutes are associated with some impediments and, therefore, remain under trial (Stear *et al.*, 2007). On the other hand, botanical dewormers are presently an interesting field of investigations predicted to be a dependable alternate to control parasites in the near future (Waller *et al.*, 2001; Jabbar *et al.*, 2006; Lateef *et al.*

al., 2013). There is plethora of reports regarding anticesticidal, antitrematocidal, antinematocidal and anticoccidial activities of plants to cure parasitism in animals (Abbas *et al.*, 2012; Sindhu *et al.*, 2012). In fact, the studies on the combination of plant extracts to control antinematocidal-resistant and susceptible gastrointestinal nematodes are extremely rare or even nonexistent. Most reports about the synergistic activity of mixed plants have been emanated from traditional Indian and Chinese Medicine (Sabu and Kuttan, 2002; Li, 2009; Munir *et al.*, 2013). Additionally, livestock raisers in Pakistan claim to have generated considerable results from using mixed plants. Needless to say, those allegations have yet to be proven scientifically. The reason for executing the current study, then, was two-fold. First fold included evaluation of synergistic activity between *N. tabacum* and *A. indica*. The other aim was determining the potency of the above combined plant extracts as compared to levamisole and ivermectin-resistant *H. contortus* in sheep.

MATERIALS AND METHODS

Site of field work: The field experiments were performed at Angora Goat Farm, Layyah District, Pakistan. There is a big gap in the annual climatic conditions in the farm due to its location in the sub-tropical region. The temperature reaches 50°C in the summer and declines to 4°C in winter. The recorded humidity is 35 to 60% in different months of the annum. The yearly rainfall varies from 150-200 mm in the summer (Monsoon season) and 50 mm in winter. The majority of sheep reared on the farm belongs to the local Pak Karakul breed. Sheep forage on paddocks during day time and, are housed and fed on dry straw at night. Green grass is seldom given and no industrial fodders or premixes are offered to animals. Benzimidazoles (oxfendazole and albendazole), levamisole (LEV) and ivermectin (IVM) are the most regularly employed antinematocidal on the farm.

Selection of experimental animals: FAMACHA Anaemia Guide Chart (Macedo *et al.*, 2010) was used for choosing 135 ram and ewe lambs (3-6 months old) which have not had any anti-worm drug administered for the last 2-3 months. The estimated eggs per gram of feces (EPG) were not less than 150 eggs per naturally infected animal (Coles *et al.*, 1992). The percentage of *H. contortus* infection was >90% (Bowman *et al.*, 2003). The animals chosen for the field trial were isolated from the flock and randomly allocated and tagged into the following nine groups (n=15 in each group):

- 1: LEV resistance detection
- 2: IVM resistance detection
- 3: *N. tabacum* low dose (2 g kg⁻¹ BW)
- 4: *N. tabacum* high dose (4 g kg⁻¹ BW)
- 5: *A. indica* low dose (2 g kg⁻¹ BW)
- 6: *A. indica* high dose (4 g kg⁻¹ BW)
- 7: Combined *N. tabacum* *A. indica* and low dose (1+1 g kg⁻¹ BW)
- 8: Combined *N. tabacum* *A. indica* and high dose (2+2 g kg⁻¹ BW)
- 9: Infested untreated

Fecal examination: Qualitative and quantitative fecal examinations were performed during the selection of all the groups. Fecal egg count reduction test (FECRT) was utilized for the diagnosis of resistance against LEV and IVM and assessment of solitary *N. tabacum* and *A. indica* in addition to the combined *N. tabacum* and *A. indica* against LEV and IVM -resistant *H. contortus* according to standard parasitological procedures (Soulsby, 1982; Coles *et al.*, 1992) and as mentioned in the previous paper of Hamad *et al.* (2013).

Coproculture: Coprocultures were also conducted to evaluate the contribution of other gastrointestinal nematodes through identification of the larvae (L3) of each nematode worm (MAFF, 1986) and as explained in the previous paper of Hamad *et al.* (2013).

Studies on antinematocidal resistance - FECRT: LEV 1.5% [Epla Lab (Pvt) Ltd] and IVM 1% [Cherished Pharmaceuticals (Pvt) Ltd] were provided by Sanna Laboratories, Faisalabad, Pakistan and checked for their accuracy percentages of active ingredients by HPLC analysis in Central Hi-Tech Laboratories, University of Agriculture, Faisalabad-Pakistan (UAF). The animals in group 1 were treated with LEV at the recommended dose (7.5 mg kg⁻¹ BW), group 2 was treated with IVM at the recommended dose (0.2 mg kg⁻¹ BW), whilst, group 9 was left as the control. Fecal examinations and coprocultures of the animals were conducted at day 14 (post-treatment) as explained in the earlier paper of Hamad *et al.* (2013).

RESO program (CSIRO Animal Health Research Laboratory, Private Bag 1, Parkville, Vic. 3052, Australia) was exploited to analyze the FECR data including arithmetic mean, variance of counts, FECR% and 95% confidence interval. As proposed by Coles *et al.* (1992), resistance is evolves if (i) the FECR% is less than 95% (ii) the lower limit of 95% confidence interval is less than 90% (iii) If merely one of the two criteria is met, resistance is suspected. Any negative value computed from FECR % and lower limit of confidence interval was reckoned equal to zero, interpreting that the resistance is extremely rampant and at the disastrous level as proposed by Gill (1996).

Potency of combined plants against resistant *Haemonchus contortus*

Plant extraction: *N. tabacum* leaves *A. indica* seed kernels were purchased from the local market of Faisalabad, Pakistan. The extraction performed following the procedure suggested by Gilani *et al.* (2004) and as stated in the former paper of Hamad *et al.* (2013).

FECRT: The animals in groups 3 and 4 were treated with *N. tabacum* CAME at low and high doses respectively; while groups 5 and 6 were treated with *A. indica* CAME at low and high doses respectively. Groups 3, 4, 5 and 6, then, served as standards to detect synergistic activity of low (group 7) and high (group 8) doses of combined *N. tabacum* and *A. indica* CAMEs. Ultimately, group 9 served as infested control without treatment. Fecal examinations and coprocultures of the animals were executed at day 14 (post-treatment) as mentioned in the previous paper of Hamad *et al.* (2013). FECR % was calculated by the formula described in the above section "Studies on antinematocidal resistance".

Egg hatch assay (EHA) was performed to assess the inhibitory impacts of various concentrations of the CAMEs of combined *N. tabacum* and *A. indica* extracts in addition to solitary of *N. tabacum* and *A. indica* (for comparison purposes) on the hatching of the *H. contortus* eggs. EHA was carried out according to the methodology depicted by Coles *et al.* (1992) with simple modifications by some investigators to be more appropriate for the evaluation of ovicidal activity of medicinal botanicals (Macedo *et al.*, 2010) and as cited in the earlier paper of Hamad *et al.* (2013). LC50 was calculated using probit analysis.

Adult motility test (AMT) was used with simple modifications to determine the efficacy of the plant CAMEs to kill or paralyze live adult resistant *H. contortus* in sheep (Singh *et al.*, 1985) and as mentioned in the previous paper of Hamad *et al.* (2013).

Statistical analysis: The RESO Computer Programme was used for detecting resistance among the parasite populations after performing FECRT (Coles *et al.*, 1992). Probit analysis was employed for the calculation of LC50 (Hubert and Kerboeuf, 1992). Completely randomized design (CRD) was applied to assess the ovicidal activity of plants through conducting EHA. For analyzing the data collected from FECRT, Tukey's test was applied and F-values were calculated. To compare between low and high doses of the used plant, t- test was applied. Analysis of data collected from AMT (an *in vitro* test), 3- factor (time, concentration, mortality) CRD was applied. Significant differences among various means were determined using Tukey's test at 5% level of significance.

RESULTS

Contribution of different nematodes in parasitized experimental animals: Coproculture disclosed that *H. contortus* had a major contribution (>90%) in infestation of experimental animals in all the groups. The animals that underwent the experiment were predominantly infested with *H. contortus*; however, other gastrointestinal nematodes poorly contributed in the infection as well (Table 1).

Resistance detection

Coproculture: Evidenced by the results of pre-treatment coproculture of mixed fecal specimens of group 1 (LEV), group 2 (IVM) and group 9 (control) of animals, the LEV, IVM and control groups had 94, 91 and 93% *H. contortus* infestation, respectively; while infestation with the other concurrent nematodes was 6, 9 and 7%, respectively (Table 1). Post-treatment (day 14), the LEV and IVM had 92 and 93% *H. contortus* infection, respectively, while the shares of other nematodes were 8 and 7%, respectively (Table 2).

FECRT: Mean EPG reduction on day 14 post-treatment with LEV and IVM were 247±56.41 SE and 213±63.53 SE, respectively (control mean EPG=987±364.26 SE). The calculated FECR% and lower confidence interval 95% for LEV, based on RESO Programme, were 75 and 38, respectively, while they were 78 and 42 for IVM,

respectively. Consequently, it was concluded that the parasite had developed resistance against LEV and IVM at Angora Goat Farm. Needless to say, however, IVM was somewhat more efficacious than LEV.

Potency of combined plant extracts against resistant *Haemonchus contortus*: After extraction, the yield of CAMEs from *N. tabacum* and *A. indica* were 1.95 and 2.34%, respectively. The results of post -treatment coproculture of pooled fecal samples of all the animals included in low and high dose groups of sole *N. tabacum* and *A. indica*, in addition to combined extracts of *N. tabacum* and *A. indica* are shown in table 2. The procured data revealed the potency of the combined extracts at high dose (2+2 g kg⁻¹ BW) which was determined by non-recovery of any larva as compared to the solitary plant extracts.

FECRT: the results of antinematocidal activity (based on mean EPG post-treatment and mean FECR%) of solitary and combined *N. tabacum* and *A. indica* CAMEs (low and high doses) against LEV and IVM -resistant *H. contortus* populations in the experimental animals naturally infested with the predominant parasite (90%) are shown in table 3. The comparison between the low and high doses of the solitary and combined *N. tabacum* and *A. indica* CAMEs (based on FECR %) is demonstrated in figure 1.

EHA: the procured data from analysis of variance (ANOVA) of EHA pertaining to ovicidal activity of different concentrations of combined *N. tabacum* and *A. indica* CAMEs, in addition to the calculation of the mean±SE of hatching inhibition (%) verified various impacts of different concentrations (dose-dependent ovicidal activity) which are expressed in figure 2. The calculated mean square was 3617.48 which is highly significant (P<0.01). The estimated LC50 value of the combined plant extracts was 0.523 µg ml⁻¹ (range 0.465-0.586), while the LC50 values of lone *N. tabacum* and *A. indica* were 0.566 µg ml⁻¹ (range 0.496-0.642) and 1.169 µg ml⁻¹ (range 1.047-1.303), respectively. According to the calculated LC50 values, the combined extracts were more effective than Sole plants, however, pharmacologically; they did not reach the level of synergism.

AMT: referring to the data perceived from the adulticidal activity of combined *N. tabacum* and *A. indica* extracts in addition to sole *N. tabacum* and *A. indica* CAMEs, using AMT and calculated F-value from ANOVA table, there was a significant difference (P<0.01) between the three factors (time, concentration and death). The mean mortality, after exposure of live resistant *H. contortus* to different concentrations of the plant extracts, was counted every two hours. The LC₅₀ values of combined and solitary CAMEs at different hours were calculated and compared (Table 4).

DISCUSSION

Antinematocidal resistance (AR) against LEV and IVM in gastrointestinal nematodes (especially *H. contortus*) of ovine has been diagnosed in a reliable way on Angora Goat Farm for first time in the present study.

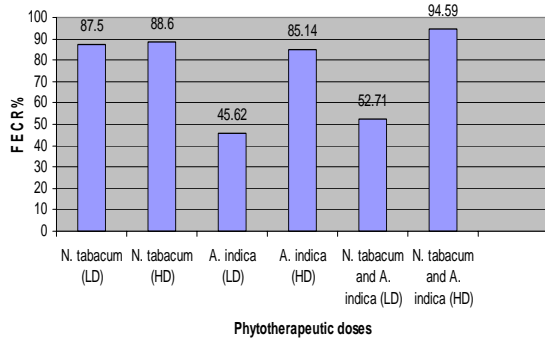


Fig. 1: Comparison between the low and high doses of the plant CAMEs (solitary and combined) for their antinematocidal efficacy in reducing the parasite egg out put percentage

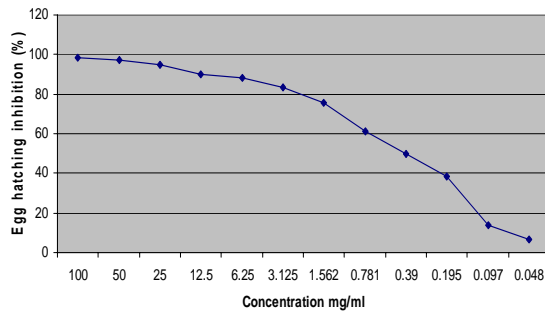


Fig. 2: Correlation between the different concentrations of combined CAMEs (*Nicotiana tabacum* and *Azadirachta indica*) and egg hatching inhibition (%)

However, the emergence of AR was also reported by some researchers in other small ruminant farms in Pakistan (Babar, 2005). The current study was intended to evaluate the efficacy of combined *N. tabacum* and *A. indica* CAMEs (50:50 w/w) to restrict rampancy of LEV and IVM-resistant *H. contortus* populations in sheep. So far, it could be ascertained that this is the first international record on potency of combined plant extracts against the antinematocidal-resistant *H. contortus* in sheep. The calculated FECR% and lower confidence interval 95% for LEV, based on RESO Programme, were 75 and 38, respectively, whilst for IVM they were 78 and 42, respectively. Accordingly, it was concluded that the parasite had developed resistance against LEV and IVM at Angora Goat Farm. IVM, however, was a bit more potent than LEV. The most important reason behind the escalation and spread of resistance against LEV and IVM in the farm is frequent yearly use of these antinematocidals which causes selection pressure on the parasite and possibly leads to the occurrence of mutation (Beech and Silvestre, 2010). It is noteworthy to mention that the annual use of LEV and IVM in the farm is 4 and 3 times, respectively. In this connection, Coles *et al.* (1995) have reported the development of AR even when just two to three drenches were given annually. On the other hand, other involving factors for evolution of AR such as bad quality of local products of antinematocidals, random use of dewormers (Afaq, 2003), non-rotation of anthelmintics, absence of any control policy on nematodes, unplanned browsing management and undernourishment should also be considered on Angora Goat Farm (Local Veterinarian File).

Table 1: Pre-treatment contribution of nematodes in the animals chosen for the study based on pooled fecal samples

Groups	<i>Haemonchus Contortus</i> L3 (%)	other nematode L3 (%)
Levamisole	94	6
Ivermectin	91	9
<i>Nicotiana tabacum</i> (L.D)	97	3
<i>Nicotiana tabacum</i> (H.D)	95	5
<i>Azadirachta indica</i> (L.D)	96	4
<i>Azadirachta indica</i> (H.D)	92	8
Combined <i>Nicotiana tabacum</i> and <i>Azadirachta indica</i> (L.D)	94	6
Combined <i>Nicotiana tabacum</i> and <i>Azadirachta indica</i> (H.D)	95	5
Control (infected untreated)	93	7

Solitary plants: L.D= Low dose (2 g kg^{-1} BW); H.D= High dose (4 g kg^{-1} BW); Combined plants: L.D= Low dose ($1+1 \text{ g kg}^{-1}$ BW); H.D= High dose ($2+2 \text{ g kg}^{-1}$ BW)

Table 2: Post-treatment percentage of *Haemonchus contortus* and other nematode L3 in the animals chosen for antinematocidal resistance and phytotherapeutic studies based on pooled fecal samples

Groups	<i>Haemonchus Contortus</i> L3 (%)	other nematode L3 (%)
Levamisole (7.5 mg kg^{-1} BW)	92	8
Ivermectin (0.2 mg kg^{-1} BW)	93	7
<i>Nicotiana tabacum</i> (LD)	poorly recovered	p. recovered
<i>Nicotiana tabacum</i> (HD)	poorly recovered	p. recovered
<i>Azadirachta indica</i> (LD)	93	7
<i>Azadirachta indica</i> (HD)	poorly recovered	p. recovered
Combined <i>Nicotiana tabacum</i> and <i>Azadirachta indica</i> (LD)	92	8
Combined <i>Nicotiana tabacum</i> and <i>Azadirachta indica</i> (HD)	not found	not found
Control (infected untreated)	-	-

Solitary plants: LD=Low dose (2 g kg^{-1} BW); HD=High dose (4 g kg^{-1} BW); Combined plants: LD=Low dose ($1+1 \text{ g kg}^{-1}$ BW); HD=High dose ($2+2 \text{ g kg}^{-1}$ BW); P= Poorly

Table 3: Mean egg per gram of feces (EPG) reduction \pm SE after treatment and fecal egg count reduction percentage (FECR %) on day 14 post-treatment with CAMEs of different plants solitary or in combination at low and high doses

CAME groups	Mean EPG post- treatment		Mean FECR%	
	LD	HD	LD	HD
<i>Nicotiana tabacum</i>	123.3 \pm 77.9*	113.3 \pm 66.1*	87.5*	88.6*
<i>Azadirachta indica</i>				
Combined <i>Nicotiana tabacum</i> and <i>Azadirachta indica</i>	536.7 \pm 367.1	146.7 \pm 58.4	45.62	85.14
Control	466.7 \pm 210.9	53.3 \pm 21.5	52.71	94.59
	987 \pm 364.3*			

*Already these data were mentioned in my published paper in Pak V J, 33: 85-90, 2013, but they are re-cited in this research article for comparison purposes only.

Table 4: Calculated LC50 (mg ml^{-1}) values for adulticidal potency of the plant extracts after conducting adult motility test

Hours	LC50 values of different plant extracts		
	<i>Nicotiana tabacum</i>	<i>Azadirachta indica</i>	combined extracts
0 hr	-	-	-
2 hr	*32.68 (19.2-66.9)	52.2 (30.1-114.0)	19.2 (11.6-36.8)
4 hr	*8.24 (5.1-14.5)	15.40 (9.99-26.0)	5.39 (3.46-8.81)
6 hr	*1.19 (0.83-1.70)	3.26 (2.34-4.60)	0.89 (0.62-1.26)
8 hr	*0.47 (0.31-0.68)	1.55 (1.09-2.19)	0.33 (0.22-0.47)
10 hr	*0.20 (0.12-0.28)	0.80 (0.59-1.08)	0.17 (0.10-0.24)
24 hr	-	-	-

0 hr = All the worms were alive; 24 hr = All the worms were dead; NB. The numbers between brackets represent lower and upper limits of 95% confidence interval; * Already these data were mentioned in my published paper in Pak V J, 33: 85-90, 2013, but they are re-cited in this research article for comparison purposes only.

Combined *N. tabacum* and *A. indica* CAMEs (high dose) demonstrated effective antinematocidal potency against LEV and IVM -resistant *H. contortus* populations in the experimental animals in all the assays (FECRT,

EHA and AMT) utilized in the study. The antinematocidal efficacy of *N. tabacum* is likely attributed to the presence of nicotine in its leaves, whilst, for *A. indica*, this character is possibly conducted to azadirachtin (a major active ingredient) which was isolated in the last century. The FECR% at low and high doses was 52.71 and 94.59, respectively. Nevertheless, there was non-significant difference ($P>0.05$) between the low and high doses within groups due to the high calculated SD and SE. On the other hand, a significant difference ($P<0.05$) was calculated between all the groups included in the trial when the F-value (2.79) was estimated from the analysis of variance table. Accordingly, the high dose of combined CAMEs was more efficacious (FECR%=94.59) than the solitary *N. tabacum* CAME at low (FECR%=87.5) and high (FECR%=88.6) doses and also from the lone *A. indica* CAME at low (FECR%=45.62) and high (FECR%=85.14) doses. In spite of these statistically salient findings, it should be mentioned that the high dose effect of the combined *N. tabacum* and *A. indica* CAMEs did not reach the level of synergism pharmacologically (Katzung, 2007). The LC₅₀ of combined CAMEs in EHA was recorded as 0.523 $\mu\text{g ml}^{-1}$, whilst for sole *N. tabacum* and *A. indica* it was 0.566 and 1.169 $\mu\text{g ml}^{-1}$, respectively. The LC₅₀ of combined extracts was ranked the top one among the others above LC₅₀ values. This result verifies that these combined CAMEs possess highest ovicidal efficacy on the hatching inhibition of *H. contortus* eggs as compared to the lone CAMEs. Regarding AMT, all worms were found dead at 6 hours post-exposure to 25 mg ml^{-1} of combined CAMEs. With diluting concentration, death of the worms was retarded. Combined CAMEs were even more efficacious than closantel; the reference drug at 25 $\mu\text{g ml}^{-1}$ that killed all the helminths 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 estimated LC₅₀ values in AMT 10 hours post-exposure were 0.17, 0.20 and 0.80 mg ml^{-1} for the combined CAMEs, lone *N. tabacum* and *A. indica*, respectively. Most reports concerning the synergy between medicinal plants have originated from conventional Indian and Chinese Medicine (Sabu and Kuttan, 2002; Li, 2009). It is necessary to say that these allegations have yet to be proven scientifically. Pertaining differences in the efficacy of *N. tabacum* and *A. indica* extracts (combined and solitary), some investigators have reported higher antinematocidal potency as compared to the results of the current study (Iqbal *et al.*, 2012). This may basically be owing to the variations among target helminths, resistance status of the parasitic nematodes, type of chemical solvent employed and source of the plant materials, etc.

Ultimately, it has been reported that certain *N. tabacum* contains quinolizidine and piperidine alkaloids which are inducing teratogenic effects in livestock when ingested during early growth stages. These alkaloid contents differ between plant species according to the season of the year, ecological conditions, stage of plant growth and part used by animals (Panter *et al.*, 2002).

Conclusion: In the light of results of this study and pursuant to the recommendations of W.A.A.V.P (second

edition), it can be concluded that the high dose of combined extracts of *N. tabacum* leaves and *A. indica* seed kernels is deemed as an effective antinematocidal. The priority could be given to this mixed extracts in stead of solitary extracts of *N. tabacum* and *A. indica* for the treatment of the parasitized sheep with antinematocidal-resistant *Haemonchus contortus*.

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