Assessment of *Azadirachta indica* seed kernel extracts to restrict the rampancy of antinematicidal –resistant *Haemonchus contortus* in ovine

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A R T I C L E   I N F O

A B S T R A C T

The current study was executed to evaluate the potency of crude aqueous methanol extract (CAME) of *Azadirachta* (*A.*) *indica* seed kernels in controlling the prevalence of oxfendazole (OXF), levamisole HCL (LEV) and ivermectin (IVM)-resistant *Haemonchus* (*H.*) *contortus* in sheep. Faecal egg count reduction test (FECRT), calculated by the RESO program, revealed an emergence of resistance among the parasite populations against the aforementioned antinematicidals particularly OXF [FECR% = -56 and lower confidence interval (LCI) = -311]. The recorded FECR% for LEV and IVM was 75 and 78 whilst the LCI for both of them was 38 and 42, respectively. Egg hatch assay (EHA) also exhibited a disastrous level of resistance in *H. contortus* toward OXF. Antinematicidal activity of *A. indica* seed kernels was assessed using FECRT, EHA and adult motility test (AMT). Mean egg per gram of faeces (EPG) recorded post-treatment of sheep at low (2g g⁻ k¹ BW) and high (4g kg⁻ ¹ BW) doses of *A. indica* CAME was 539±367.11 SE and 147±58.45 SE, respectively, as compared to the control group (mean EPG= 987±364.26 SE). There was no significant difference statistically (P>0.05) in FECR% (45.62 *vs* 85.14) in sheep at low and high doses of the plant. The results of AMT revealed a dose and time dependent efficacy of CAME of the assessed plant to kill antinematicidal-resistant adult worms with calculated LC_{50} values of 52.20, 15.40, 3.26, 1.55 and 0.80 mg ml⁻¹ after 2, 4, 6, 8 and 10 hours. Accordingly, high dose (4g kg⁻ ¹ BW) of *A. indica* seed kernel extract could be used to treat sheep carrying antinematicidal- resistant populations of *H. contortus*.

1. INTRODUCTION

Small ruminants are vastly exposed to infestation with different genera and species of gastrointestinal (GI) nematodes in tropical, subtropical and even in moderate climate zones in the world (Getachew *et al*., 2007). Categorically, the abomasal inhabitant, blood-sucking *Haemonchus* (*H.*) *contortus* is considered more dangerous to ovine and caprine as compared to other pathogenic nematodes due to its highly detrimental impacts on livestock productivity (Urquhart *et al*., 2007; Bowman, 2009). To control nematodiasis, organized sheep farms in developing countries and comparatively industrialized countries rely regularly on broad-spectrum synthetic chemotherapeutics such as benzimidazoles, imidazothiazoles and macrocyclic lactone derivatives (Ghisi *et al*., 2007). By virtue of these traditional

dewormers, the epidemicity of these alimentary worms has been patently contained. Further, the emergence of antinematicidal resistance (AR) was the consequence of the recurrent annual use of these conventional antinematicidals (Neveu *et al*., 2007). Additionally, these drugs can contaminate the environment and affect public health through their residues in the food chain (Waller, 2006).

It is noteworthy to mention that the prevalence of AR has emboldened researchers to adopt and promote non-chemical alternatives of orthodox drugs. In this regard, some substitutes such as biological control, genetic approaches, immunization, nutritional supplementations and grazing management have been recommended. However, these alternate sources have some drawbacks which limit their commercialization (Stear *et al*., 2007).

On the other hand, under the umbrella of non-chemotherapeutic approaches, phytotherapy is presently an interesting area of investigation and scientific validation anticipated to be a promising alternative to control parasitism in the near future (Jabbar *et al*., 2006). Medicinal plants have been exploited for thousands of years to cure human and animal ailments as part of ethno-medicine and ethno-veterinary practices (Wanzala *et al*., 2005). In recent decades, several hundred researches have been conducted to explore the active constituents of ethnobotanicals and evaluate their efficacy against ecto and endo parasites of livestock (Macedo *et al*., 2010). The present study was carried out for assessing the potency of *Azadirachta* (*A*.) *indica* seed kernels toward antinematicidal- resistant *H. contortus* in naturally infected sheep for the first time. This phytomedicine is extensively growing in the Indo-Pakistan subcontinent and it has been known for many centuries for possessing several therapeutic activities

including antiparasitic properties (Dhawan and Patnaik, 1993). The major active ingredient of *A. indica* is azadirachtin which was isolated in the last century (Butterworth and Morgan, 1968). Previously, some studies have been carried out to assess this plant for its antinematicidal activity (Akhtar and Riffat, 1984; Hördegen *et al*., 2003; Costa *et al*., 2006; Costa *et al*., 2008). These studies; however, have been conducted randomly without giving attention to the efficacy of ethnobotanicals against known antinematicidal-resistant nematodes. Antinematicidal-resistant nematodes are more pathogenic and prolific, have ameliorated inhabitancy rates in the host and an enhanced prolonged existence of the free-living stages on paddock (Kelly *et al*., 1977). Therefore, crude aqueous methanol extract of this phytomedicine was evaluated utilizing *in vivo* and *in vitro* parasitological assays.

2. Material and Methods

Animals (n=90) aged 3-6 months with egg per gram of feces (EPG) of more than 150 eggs, that had not been dewormed for the last 8-12 weeks, were chosen for this study (Coles *et al*., 1992). FAMACHA Anaemia Guide Chart was used to facilitate the selection of animals (Macedo *et al*., 2010). The percentage of infection with *H. contortus* as compared to other nematodes was >90% in each animal (Bowman *et al*., 2003). The selected animals for the study were isolated from the flock and randomly allocated and tagged into the following six groups:

Group 1: Oxfendazole resistance detection group (n=15)

Group 2: Levamisole HCL resistance detection group (n=15)

Group 3: Ivermectin resistance detection group (n=15)

Group 4: *A. indica* low dose (2 g kg^{-1} BW) treated group (n=15)

Group 5: *A. indica* high dose (4 g kg^{-1} BW) treated group (n=15)

Group 6: Infected untreated group (n=15)

Each group of the experimental animals was penned alone and fed on rough fodder without administering any kind of therapy during the experiment.

NB. One control group was used for both detection of antinematicidal resistance and evaluation of the plant trials because we statistically dealt with one population.

• Diagnosis of *Haemonchus contortus*
Faecal examination

Qualitative and quantitative faecal examinations of the animals were performed during the selection process as a preliminary detection of nematodiasis. Fecal specimens were taken from each animal individually and other steps were carried out according to Coles *et al*. (1992). Where possible, the nematode eggs were identified using the diagnostic key of Soulsby (1982).

Coproculture

Coprocultures were also executed to determine the involvement of various species of nematodes in overall natural worm infestations MAFF (1986) during the selection process. Fecal specimens from each group of experimental animals were pooled and cultured in glass dishes. The cultures were incubated for seven days at 27±1°C. The larvae (L$_3$) were, then, collected using the Baermann apparatus. Lugol's iodine was added to the cultures and 100 larvae were counted and identified according to MAFF (1986).

• Detection of antinematicidal resistance
FECRT: Oxfendazole (OXF) 2.265%, levamisole (LEV) 1.5% [Epla Lab. (Pvt.) Ltd.] and ivermectin (IVM) 1% [Cherished Pharmac. (Pvt.) Ltd.] were procured from Sanna Laboratories for Pharmaceuticals and Vaccines, Faisalabad-Pakistan and analyzed for their authenticity by HPLC analysis in Central Hi-Tech Laboratories, University of Agriculture- Faisalabad, Pakistan (UAF). The animals in group one, were treated with the recommended doses of OXF (5 mg kg^{-1} BW), group two was treated with LEV (7.5 mg kg^{-1} BW), and group three was treated with IVM (0.2 mg kg^{-1} BW) while group six was left as infected (untreated control). Fecal examinations and coprocultures of the animals were performed at day 14 (post-treatment) as depicted previously. Records of post OXF, LEV and IVM treatment EPG in addition to control group EPG and composition of nematode infections were kept. The following formula was used to calculate (FECR %):

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

RESO program (CSIRO Animal Health Research Laboratory, Private Bag 1, Parkville, Vic. 3052, Australia) was utilized 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 the FECR% is below 95% and the lower limit of 95% confidence interval is below 90%. If only one of the two criteria is met, resistance is suspected. Any negative values calculated from the FECR% and lower limit of confidence interval were deemed equal to zero, indicating that the resistance is highly rampant and at the catastrophic level as proposed by Gill (1996).

EHA was carried out following the standardized protocol that was accepted by the World Association for the Advancement of Veterinary Parasitology (W.A.A.V.P) and described in details by Coles *et al.* (1992) to diagnose resistance against OXF.

The following formula was used for the calculation of hatching inhibition (%):

Hatching inhibition (%) = P test/ P total $\times 100$

P test: number of unhatched or embryonated eggs.

P total: number of unhatched or embryonated eggs + Larvae (L_1)

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 deemed as an indication of antinematicidal (OXF) resistance as suggested by Coles *et al.* (1992).

• **Assessment of *Azadirachta indica* extracts against *Haemonchus contortus***

Plant extraction

A. indica seed kernels were purchased from the local market of Faisalabad-Pakistan. The plant seed kernels were finely pulverized to a powder in an electric grinding machine and kept in cellophane bags. The ground plant materials were soaked in 70% aqueous methanol (the concentration adjusted by an alcohol meter) by cold maceration at room temperature with the mixture stirred twice daily. After three days, the filtrate was collected through a piece of porous cloth and the plant materials re-soaked in 70% aqueous methanol. This process was repeated three times. The combined filtrates were concentrated in a rotary evaporator at 40°C under reduced pressure. 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 exploited against the parasite using *in vivo* and *in vitro* techniques. The percentage yield of extracts was calculated as under:

Ratio= A/B$\times$100

A: weight yield after extraction (g)

B: dry matter weight (g)

FECRT: The animals in groups 4 and 5 were treated with *A. indica* CAME at low (2 g kg^{-1} BW) and high (4 g kg^{-1} BW) doses, whereas

group 6 served as infected, untreated control. Fecal examinations and coprocultures of the animals were executed at day 14 (post-treatment) as mentioned above. Records of 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 the hatching of the parasite eggs. 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 the evaluation of plants (Macedo *et al*., 2010).

AMT was used with minor modifications to determine the effect 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 collected 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 different concentrations (100-0.048 mg ml⁻¹) in a 24-well flat-bottomed titration plate. The positive control well received 25 μg ml⁻¹ of closantel dissolved in PBS, while 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 placed in each well containing CAME, positive and negative controls. The worms were observed at 0, 2, 4, 6, 8, 10 and 24 hours for their motility, paralysis and mortality. There were three replicates for each concentration.

3. Results and Discussion

3.1. Results

• Composition of natural nematode infections in experimental animals

The experimental animals were predominantly infested with *H. contortus*. However, other nematode species were also concomitant such as: *Teladorsagia* spp., *Chabertia* spp., *Oesophagostomum* spp. and *Trichostrongylus* spp. According to the coproculture, *H. contortus* was the main contributor to the infection (>90%) of the experimental groups of animals (Table 1).

• Resistance studies

Coproculture

The proportions of infection with *H. contortus* and other coexisting nematode species pre-treatment and post-treatment (day 14) with OXF, LEV and IVM other than control group, after performing coproculture of pooled fecal samples of the animals in each group, are presented in table 1 above.

FECRT: Mean EPG reduction and FECR% on day 14 (post-treatment with OXF, LEV and IVM) analyzed by the RESO program in

addition to the calculation of lower confidence interval 95% (table 2) had indicated that the resistance was at disastrous levels in the farm, particularly in the case of OXF.

EHA: The LC_{50} of OXF was found to be 1.86 µg ml^{-1} (range 1.45-2.45), which was in excess to 0.1 µg ml^{-1} proposing evolution of resistance against *H. contortus* (Coles *et al*., 1992). Correlation between ovicidal activity of different concentrations of OXF and hatching inhibition (%), through executing EHA, is exhibited in figure 1.

- **Efficacy of *Azadirachta indica* against resistant *Haemonchus contortus***

The yield of CAME from *A. indica* seed kernels was 2.34%. Post -treatment coproculture of pooled fecal samples of the animals included in the high dose group of *A. indica* revealed very poor recovery of *H. contortus* and other nematode larvae (L_3). In the low dose group, the recorded percentage of *H. contortus* and other nematode larvae (L_3) was 93% and 7%, respectively. In the control group, the percentage of *H. contortus* L_3 was 95% while recovered L_3 of other nematodes was 5% (table 1).

FECRT: the results of antinematicidal activity of *A. indica* CAME (low and high doses) against OXF, LEV and IVM- resistant *H. contortus* populations in the experimental animals naturally infected with the predominant parasite (>90%) as well as the comparison between impacts of low and high

doses of the plant extracts on the mean of egg reduction is statistically analyzed (table 3). The results of coproculture after treatment, the group treated with high dose of *A. indica* extracts (FECR% > 80), clearly shows that very few larvae of *H. contortus* and other nematodes were recovered (table 1).

EHA: the procured data from the analysis of variance (ANOVA) of EHA regarding ovicidal efficacy of different concentrations of *A. indica* CAME, other than the calculation of the mean±SE of hatching inhibition (%), revealed diverse influences of different concentrations (dose-dependent ovicidal activity) (figure 2).

The calculated mean square was 3748.25, which is highly significant (P<0.01). The calculated LC_{50} was 1.169 mg ml^{-1} (range 1.047-1.303).

AMT: pursuant to the data procured from the adulticidal efficacy of *A. indica* CAME utilizing AMT and computed F-value from the ANOVA table, there were significant differences (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 recorded every two hours. The data is displayed in figures 3. The LC_{50} values at different hours are also calculated (table 4).

3.2. Discussion

Resistance has developed against conventional antinematicidals (OXF, LEV and

IVM) by GI nematodes particularly *H. contortus* in most countries of the world (Bartley *et al*., 2004; Neveu *et al*., 2007) including Pakistan (Babar, 2005; Saddiqi, 2005; Hamad *et al*., 2013). Due to the absence of dependable alternate sources to synthetic chemotherapeutics (Stear *et al*., 2007) researchers, especially in the Indo-Pakistan subcontinent, Africa and South Latin America have promoted indigenous medicinal plants as an effective substitute to fight parasitism (Waller *et al*., 2001; Cala *et al*., 2012). Hence, this study was conducted to assess the antinematicidal activity of *A. indica* seed kernels against antinematicidal-resistant *H. contortus* for the first time in the world.

For OXF, the calculated FECR% was (-56) while the LCI was (-311) signifying that resistance was at the disastrous level (Gill, 1996) in the farm because even a triple dose (15 mg kg^{-1} BW) was ineffective to minimize the parasitic burden in the infected sheep (local veterinarian file). LC_{50} value of OXF (1.86 µg ml^{-1}), calculated by the probit analysis after performing EHA, also indicated a rampancy of resistance among the *H. contortus* populations. The calculated LC_{50} value was higher than 0.1 µg ml^{-1}; an indication of OXF resistance (Coles *et al*., 1992). Accordingly, it can be concluded that the resistance level among *H. contortus* populations in sheep is more than 25%, which means that the parasite is highly resistant to OXF. In this regard, the antinematicidal resistance could not be diagnosed by traditional parasitological methods if the resistance level is below 25% among the GI nematodes (Martin *et al*., 1989). Antinematicidal resistance to LEV and IVM was detected in the farm as well. The calculated FECR% was 75 and 78; respectively while the LCI was 38 and 42 according to the RESO program. It may be concluded that the parasite is more susceptible to LEV and IVM as compared to OXF. However, even a 1.5 dose (standard dose is 7.5 mg kg^{-1} BW) of LEV and a double dose (standard dose is 0.2 mg kg^{-1} BW) of IVM were ineffective (local veterinarian file) due to the escalation of resistance among the parasitic populations. The main reasons for the evolution and prevalence of multiple forms of resistances in the farm are the discriminate and recurrent annual uses of these drugs. The local veterinarian file showed that OXF is used five times annually, LEV is administered four times per a year, and IVM is drenched three times per annum. Coles *et al*. (2005) have indicated the development of antinematicidal resistance even when only two to three drenches were administered annually.

CAME of *A. indica* seed kernels revealed antinematicidal efficacy against OXF, LEV and IVM -resistant *H. contortus* in sheep in all the assays (FECRT, EHA and AMT) used in this study. The main active constituent of *A. indica* seeds is azadirachtin (Butterworth and Morgan, 1968), which possibly plays a big role in killing resistant *H. contortus*. Although there was a significant difference in reduction of

EPG post-treatment with low (539±367.11 SE) and high (147± 58.45 SE) doses of the plant CAME, the FECR% was non-significant ($P > 0.05$) in the animals treated at low (45.62) dose as compared to high (85.14) dose of CAME. The reason for the non-significant difference between low and high doses is attributed to the high calculated standard deviation and standard error. The LC_{50} of CAME in EHA was calculated as 1.169 μg ml $^{-1}$ (range 1.047-1.303 μg ml $^{-1}$). In AMT, all helminths were observed dead at 10 hours post-exposure to 25 mg ml $^{-1}$ of CAME. With decreasing concentration, death of the worms also declined. In accordance with the time required to kill 100% of live resistant *H. contortus*, the plant CAME at 25 mg ml $^{-1}$ was similar to closantel (positive control) in killing the parasite; 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 helminths were observed dead after 24 hours. The LC_{50} of CAME in AMT was calculated as 52.20 mg ml $^{-1}$ (range 30.14-114.02 mg ml $^{-1}$) two hours post-exposure, while the calculated LC_{50} value was 0.80 mg ml $^{-1}$ (range 0.59-1.08) after 10 hours of exposure. Phytomedicines have been identified as antiparasitics (Waller *et al*., 2001). The presence of multiple natural phytochemicals in plants categorizes them as wide spectrum antiparasitics. Exploitation of plants to treat different parasitic diseases is justified,

particularly in the rural areas of developing countries, where ailments like parasitism (Farooq *et al*., 2012) have had an enormous negative effect on animal productivity due to the inaccessibility to allopathic drugs.

In contrast to the current study, some researchers have recorded higher antinematicidal potency of *A. indica* (Iqbal *et al*., 2006). These variations may, principally, be owing to the differences among target helminth populations, resistance status of the parasitic nematodes, and source of the plant. Other contributing factors, like the biological activity of plants which depends on the source of the plant, cropping season (Hammond *et al*., 1997), mode of calculation of the dosage, variation within species of plants, storage techniques and drying processes (Croom, 1983) should also be considered. Furthermore, tther reasons that justify this variation are that resistant parasites are more fecund, pathogenic and have increased settlement rates in parasitized hosts (Kelly *et al*., 1977).

Pursuant to the recommendations of W.A.A.V.P (second edition) edited by Wood *et al*. (1995), an anthelmintic with FECR% (98) is deemed highly effective; FECR% (80) and above is effective while FECR% less than 80 is not recommended. In accordance with the present study and W.A.A.V.P categorization, *A. indica* seed kernels are classified as an effective antinematicidal.

Table 1: Pre-treatment and post-treatment contributing nematodes in the animals chosen for the study based on pooled faecal specimens

Experimental Groups	Pre-treatment L$_3$ (%)		Post-treatment L$_3$ (%)	
	H. contortus	other nematodes	*H. contortus*	other nematodes
Oxfendazole	96	4	97	3
Levamisole	94	6	92	8
Ivermectin	91	9	93	7
A. indica (Low dose)	96	4	93	7
A. indica (High dose)	92	8	poorly recovered	poorly recovered
Control (Not-treated)	93	7	-	-

Table 2: Mean EPG and FECR% on day 14 post-treatment in addition to resistance status of the synthetic drugs calculated by RESO program

Treatment Groups	Mean EPG±SE	Confidence interval		FECR%	Status of drug resistance
		Upper	Lower		
*Oxfendazole	1543±432.41	40	-311**	-56**	Highly resistant
Levamisole	247±56.41	90	38	75	Resistant
Ivermectin	213±63.53	92	42	78	Resistant
Control	987±364.26	-	-	-	-

* Although, my previous paper published in Pakistan V. J. 33: 85-90, (2013) has embraced these data, but it is necessary to re-cite them in this research article.

** Negative values mean zero

Table 3: Statistical analysis of results obtained post-treatment with low and high doses of the plant CAME

Treatment	Mean EPG reduction Post-treatment	±SD	±SE	t-value	probability	Mean FECR%
Low dose (2 g kg^{-1} BW)	539	1421.83	367.11			45.62
				1.05 [NS†]	0.303	
High dose (4 g kg^{-1} BW)	147	226.36	58.45			85.14
Control (Mean EPG=987±364.26 SE)						

[†]Non-significant difference (P>0.05) between low and high doses

Table 4: Calculated LC50 values for adulticidal activity of *Azadirachta indica* after performing adult motility test

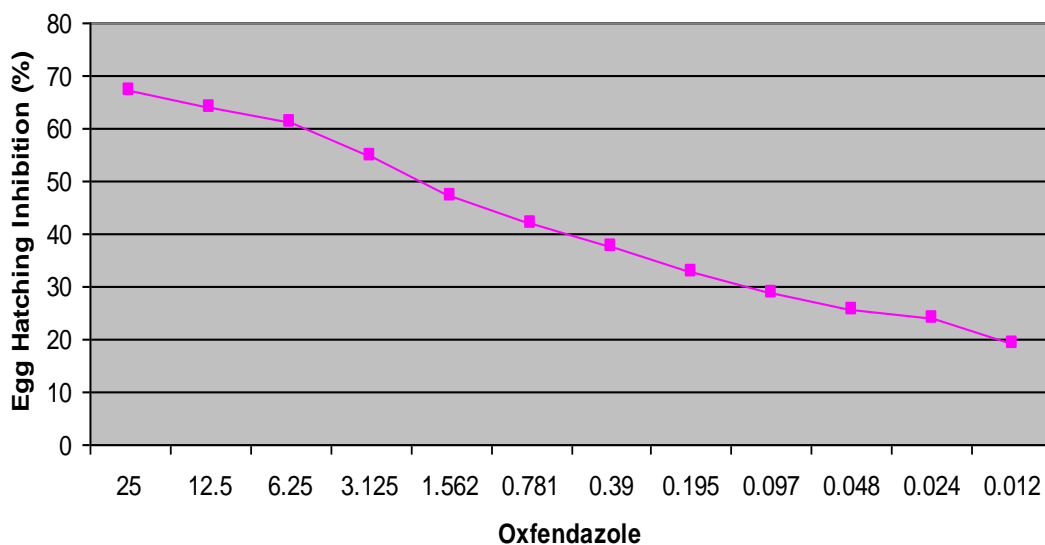| Hours (Post-exposure)	LC$_{50}$ (mg ml^{-1})	95% Confidence interval	
		Lower	upper
0 hr	-	-	-
2 hr	52.20	30.14	114.02
4 hr	15.40	9.99	20.05
6 hr	3.26	2.34	4.60
8 hr	1.55	1.09	2.19
10 hr	0.80	0.59	1.08
24 hr	-	-	-

All parasites were alive at 0 hr and dead at 24 hr

Figure1: Correlation between ovicidal activities of different concentrations of oxfendazole (µg ml⁻¹) and hatching inhibition (%) through conducting egg hatch assay
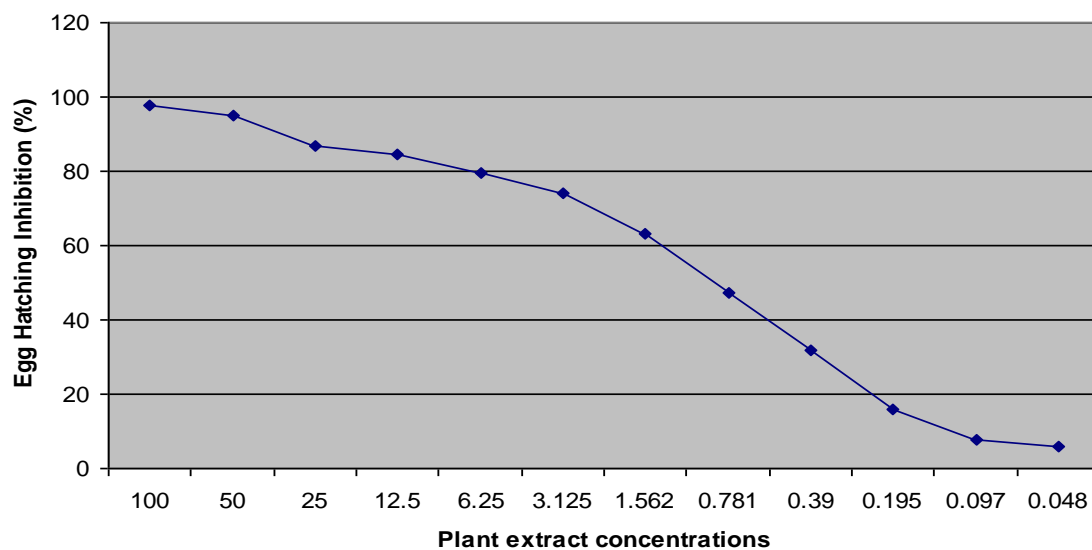

Figure 2: Correlation between ovicidal activities of different concentrations (mg ml⁻¹) of *Azadirachta indica* extracts and egg hatching inhibition (%) through carrying out egg hatch assay
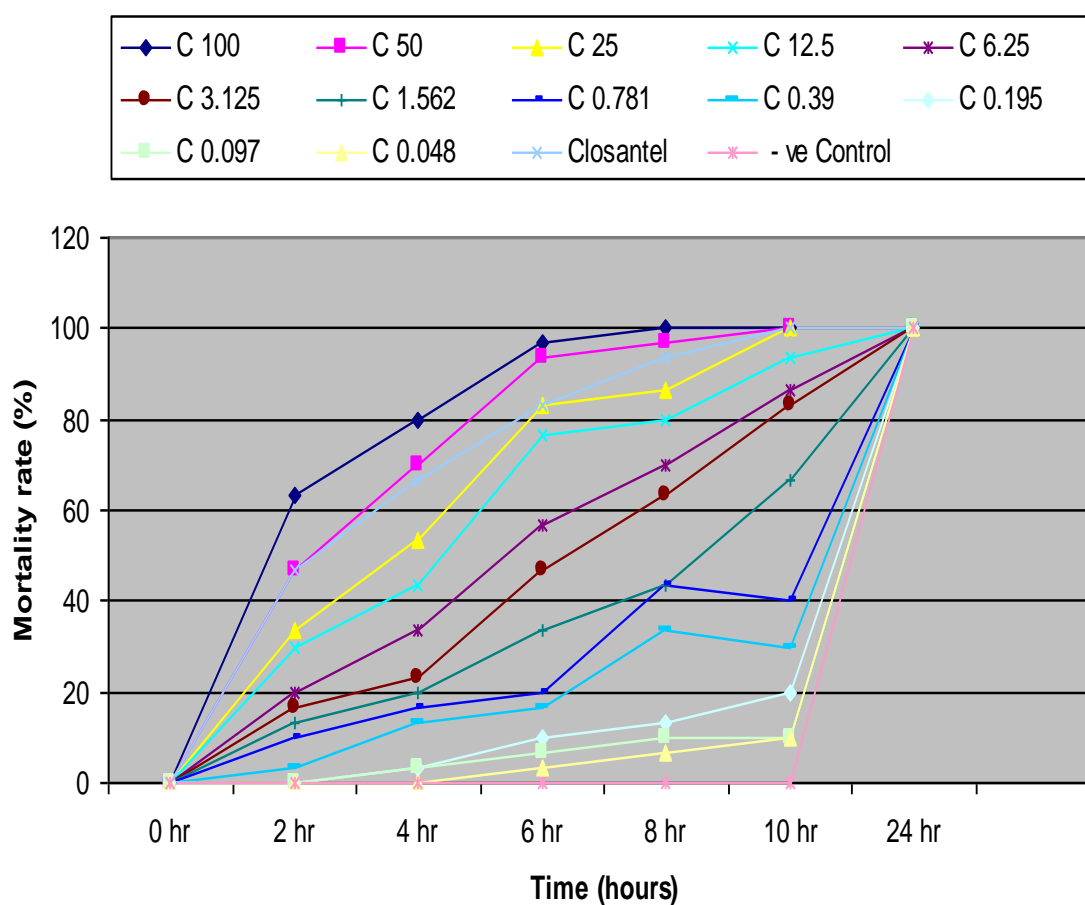
Figure 3: Mortality (%) of resistant live adult *Haemonchus contortus* post-exposure to the CAME of *Azadirachta indica*

C= Concentration of each CAME (mg ml⁻ ¹)

Closantel concentration=25 µg ml⁻¹ (dissolved in PBS)

Control= I ml of 70% acetone plus 1 ml of PBS

4. Conclusions

In light of the results of this study, it may be concluded that resistance is prevalent among *H. contortus* populations in sheep against OXF, LEV and IVM in Angora Goat Farm. Antinematicidal resistance is at a catastrophic level in the case of OXF. The reasons behind the development and rampancy of multiple drug resistance in the farm are the indiscriminate and recurrent annual administering of antinematicidals. High doses (4g kg^{-1} BW) of CAME of *A. indica* seed kernels is effective in the treatment of sheep harboring OXF, LEV and IVM-resistant *H. contortus* populations. Thus, the prevalence of multiple drug resistance could be controlled significantly using *A. indica* seed kernels.

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Conflict of interest statement

The author of this research article attests to no conflicts of interest concerning the data incorporated in this document.

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