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Evaluation of anthelmintic potential of aqueous leaf extract of *Azadirachta indica* in benzimidazole-resistant *Haemonchus contortus* of sheep

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Abstract
This study aimed to evaluate the anthelmintic potential of aqueous leaf extract of *Azadirachta indica* against benzimidazole-resistant *Haemonchus contortus* of sheep. A faecal egg count reduction test (FECRT) was conducted in organized sheep farms in Salem, Karur, Kanniyakumari, Kancheepuram and Thiruvallur districts of Tamil Nadu using fenbendazole. 560 dung samples collected from the above sheep farms were subjected to coprological examination revealed *Haemonchus contortus* eggs. FECRT and allele-specific PCR (AS-PCR) confirmed the benzimidazole resistance in samples from Kancheepuram and Thiruvallur districts. Aqueous extracts of *A. indica* leaves at different concentrations *viz*., 0.5, 1, 2 and 5 per cent, were tested against resistant strongyle nematodes of sheep reared in these farms with BZ resistance. Maximum inhibition of egg hatch was noticed in 5% aqueous extracts of *A. indica* (35.42±1.87 %) compared to other concentrations. The maximum mean larval paralysis observed was 28.89±1.11% in 5 % aqueous extract at 60 min. It was observed that increased concentration of extracts resulted in increased efficacy of inhibition on egg hatch and mean larval paralysis. It is concluded that *A. indica* could be a promising phytomedicine for the benzimidazole-resistant nematodes of sheep.

Keywords: Neem, *Azadirachta indica*, sheep, benzimidazole, aqueous leaf extracts, anthelmintic resistance

1. Introduction
Anthelmintic resistance poses a significant economic loss to the sheep industry, due to the increased parasitic burden leads to poor growth rates, delayed maturity, decreased reproductive performance and wool production, anaemia, diarrhoea, and death. The nematode parasites of sheep *viz.*, *Trichostrongyles* sp., *Haemonchus contortus*, and *Telodorsagia circumcincta* have been reported to have developed benzimidazole resistance by single nucleotide polymorphism (SNP). The anthelmintics have been used indiscriminately under intensive sheep farming systems, which resulted in anthelmintic resistance. The development of anthelmintic resistance leads to the search for new compounds to control helminthiasis in sheep. The sustainable and eco-friendly approaches *viz*., targeted selective therapy, biological control, and use of phytomedicines, have been recommended worldwide in the recent past. Reports documented the usage of many plant materials for treating animal diseases, especially against the gastrointestinal parasites of ruminants viz., *Pithecellobium dulce, Momordica charantia, Carica papaya, Melia azedarach, Azadirachta indica, Moringa olifera, Vitex negundo* and Oyster mushrooms (Rastogi *et al.*, 2009; Edith *et al*., 2022; Edith *et al*., 2023; Khan *et al*., 2024) [1-4]. Neem (*Azadirachta indica*) is known as the "village pharmacy. Several reports reviewed the pharmacological activities of *A. indica* (Maithani *et al*., 2011; Dubey and Kashyap, 2014; Uzzaman, 2020) [5-7] against helminthiasis.
Various parts of the Neem have been used as an anthelmintic in livestock *viz*., leaves (Costa *et al*., 2006; Chagas *et al*., 2008; Dongre *et al*., 2014; Jamra *et al*., 2015) [8-11], seed (Iqbal *et al*., 2010) [12], aqueous, ethanolic and methanolic extracts of leaves

(Priscilla *et al*., 2014; Agarwal and Bagai, 2014; Nawaz *et al*., 2014; Agarwal *et al*., 2016; Gadhave *et al*., 2019; Rehman *et al*., 2023; Hawsah *et al*., 2023; Adams *et al*., 2023) [13-20], root and stems (Yakuba *et al*., 2006) [21] and essential oils (Jeyathilakan *et al*., 2010; Batool *et al*., 2023) [22-23]. The above studies have investigated the anthelmintic effects potential of *A. indica* against gastrointestinal helminths. The knowledge gap was recognized in the potential of neem leaves of *A. indica* on benzimidazole-resistant *H. contortus.* Hence, this study was conducted to evaluate the anthelmintic potential of *A. indica* on benzimidazole-resistant *H. contortus.*

2. Materials and Methods

2.1 Collection and processing of the plant

A. indica leaves were collected from fields of Salem, Karur, Kancheepuram and Thiruvallur districts. The leaves were thoroughly washed in water. Then they were shadow-dried in the open. After complete drying, leaves were ground using an electric blender to make into powder. The powder was stored in airtight containers until further use (Figure 1a and 1b).

2.2 Preparation of aqueous leaf extract of *A. indica*

Four hundred grams of powder of *A. indica* leaves were macerated using one litre of water for 48 hours at room temperature with intermittent stirring. After maceration, double filtration was done using muslin cloth and Whatman No 1 filter paper. The filtrate obtained was evaporated using an incubator. Dried extract was stored in micro-centrifuge tubes at -20 °C until further use.

Fig 1a: *Azadirachta indica* plant leaves

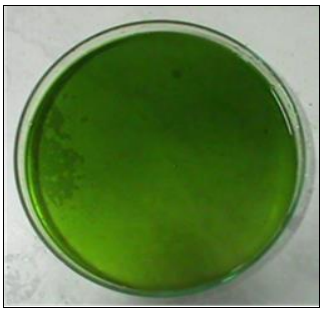


Fig 1b: Dried *A. indica* leaf extract powder

2.3 Detection of Anthelmintic Resistance

In this study, the anthelmintic resistance was detected by *in vivo* methods (faecal egg count reduction test, allele-specific polymerase chain reaction) and *in vitro* methods (Egg hatch assay and larval paralysis assay).

2.3.1 Faecal Egg Count Reduction Test (FECRT)

FECRT is the commonly used *in vivo* technique to detect anthelmintic resistance and to evaluate the anthelmintic efficacy of various compounds by comparing faecal egg counts of animals before and after treatment.

The sheep in the organized farms from Karur, Melmaruvathur, Nagercoil, Salem, Kancheepuram and Thiruvallur treated with oral fenbendazole at a standard dose rate of **7.**5 mg/Kg body weight and the dung samples were collected on 0, 7 and 14 days post-treatment.

The collected dung samples were packed in polythene bags with the air excluded and sealed. In this study, care was taken to transport the dung samples and store them in the laboratory at 4 °C before being subjected to Egg Per Gram (EPG), egg hatch assay (EHA) and larval paralysis assay (LPA) as quickly as possible.

Egg Per Gram (EPG) was calculated using Mc Master Method. Briefly, four grams of dung sample was mixed with 26 ml of tap water and homogenised. The homogenized suspension was filtered through a sieve. The collected filtrate was centrifuged at 2000 rpm for 2 min and the supernatant was discarded. 26 ml of saturated salt solution was added to the sediment and agitated. This suspension was loaded into a three-chambered McMaster slide chamber. Samples from different places were loaded separately in each chamber of Mc Master Slide. The number of eggs counted was multiplied by 25 to calculate the EPG.

2.3.2 Detection of benzimidazole resistance by allele-specific PCR

Eggs separated from dung samples were used for DNA extraction using a commercial stool DNA extraction Kit.

Amplification of β-tubulin was done in a final volume of 25 µl using primers Pn1- 5’ GGC AAA TAT GTC CCA CGT GC3’ and Pn2- 5’ GAT CAG CAT TCA GCT GTC CA3’, each 1.5 µl, 12.5µl Red dye mix, 7.0 µl DNA template and 2.5 µl of nuclease-free water. PCR reaction was carried out on a gradient thermo cycler (Eppendorf) with initial denaturation at 94 ºC for 3 min, followed by 36 cycles (denaturation 94ºC for 55 sec, annealing 57 ºC for 55 sec and extension 72 ºC for 55 sec) with the final extension at 72 ºC for 10 min and the PCR products were visualized after gel electrophoresis using Gel Doc system.

Nested PCR was performed as per the method described by Silvestre and Humbert, 2000 [24]. Briefly, the amplicons of β-tubulin were used as DNA template in a final volume of 25 µl with primers (10 pm/ µl) F Pn3-GGA ACA ATG GAC TCT GTT CG 3’ and R Pn4-GGG AAT CGA AGG CAG GTC GT 3’ each 1.5 µl, 12.5 µl Red dye mix, 3.0 µl DNA Template and 6.5 µl nuclease free water. PCR reaction was carried out on a Gradient Thermo cycler. PCR cyclic profiles and visualization were similar to β tubulin amplification.

Coproculture revealed predominantly *Haemonchus contortus* larvae. Hence this allele-specific PCR was done to detect the resistant and susceptible alleles of *H. contortus*. Reaction was performed using different sets of primers for resistant and susceptible alleles.

Amplicons of Nested PCR were used as a DNA template for this allele-specific PCR with the following published primer sequences (Silvestre and Humbert, 2000) [24].

- Ph 1-5’ GGA ACG ATG GAC TCC TTT CG 3’
- Ph2-5’ GAT CAG CAT TCA GCT GTC CA 3’
- Ph3-5’ CTG GTA GAG AAC ACC GAT GAA ACA TA 3’
- Ph4-5’ ATA CATG AGC TTC GTT GTC AAT ACA GA 3’

The reaction was performed in a total volume of 25 µl using a Gradient thermo cycler. Cyclic conditions are similar to those of beta-tubulin amplification.

PCR products were visualized by submarine gel electrophoresis and staining with ethidium bromide. Briefly, the ladder was reconstituted with deionised water, 6X gel loading dye and 200- 2000bp DNA ladder, respectively in the ratio of 4:1:1. A two per cent agarose gel was prepared by dissolving one g of agarose in 50 ml of 0.5X TBE buffer. One µl of ethidium bromide working solution was added to give a final concentration of 0.5 µg of ethidium bromide per ml of the gel. Gel was cast in UV UV-transparent submarine gel electrophoresis system (Broviga), and wells were prepared by placing a comb at one end. After complete setting of the

gel, the comb was removed and 0.5X TBE buffer was poured to cover the gel. Ten microlitres of the amplicons were mixed with 2 µl of gel loading dye and loaded into the wells. Three µl of 200-2000bp DNA ladder was included in each run. Electrophoresis was carried out at a constant voltage of 85 V. The products were visualized and photographed using a video gel documentation system (BIO RAD Gel Doc XR+).

2.3.3 Assessment of resistance by egg hatch assay (EHA)

The EHA is an economical and rapid test to assess the susceptibility of mixed nematode populations. This test calculates the efficacy of an anthelmintic by determining the ratio of the eggs that fail to hatch in solutions of increasing drug concentrations compared to control wells.

2.3.3.1 Preparation of aqueous *A. indica* extracts

Aqueous leaf extract powder of *A. indica* was dissolved in distilled water to prepare the final concentration of 0.5%, 1%, 2% and 5% in the 24-well plate.

2.3.3.2 Preparation of Thiabendazole (TBZ) stock and working solutions

The sock solution of 1000 ppm TBZ was prepared by taking 50 gm of pure TBZ (Sigma-T-8904) into a 50 ml beaker and dissolved in 50ml of dimethyl sulfoxide (DMSO), Working solutions to a final concentration of 0.1, 0.2, 0.3 µg/ml were prepared by dissolving stock solution in DMSO to a final concentration of 0.1, 0.2, 0.3 µg/ml.

The method described by Lourderaj (2005) [25] was adopted for the egg hatch assay with minor modifications. Briefly, 500µl of egg suspension containing approximately 40 eggs was added to each well of 24 multiwell plates. 500 µl of working solution of 0.1, 0.2, 0.3, µg/ml TBZ was added to each benzimidazole control well. 500µl of aqueous leaf extract of *A. indica* was added with a final concentration of 0.5%, 1%, 2% and 5% in different wells. 500 µl of DMSO (99 per cent) was added to the negative control wells. The tests were carried out with two replicates for each drug concentration and controls. The volume in each well was made up to 2 ml using distilled water. The plate was incubated at 25 °C for 48 hours. After incubation, one drop of Lugol's iodine was added to stop further embryonation of eggs. The mean number of eggs and larvae at each concentration of TBZ was counted using a binocular stereo zoom microscope (Olympus SZ40, Japan). The anthelmintic resistance was determined by the percentage of hatch and survival of larvae in the anthelmintic solution.

$$\text{Percentage hatch} = \frac{\text{Number of larvae hatched}}{\text{Total number of eggs added}} \times 100$$

The susceptibility of the helminth to the aqueous leaf extract of *A. indica* was determined based on the percentage of unembryonated eggs after 48 hrs of incubation. The number of embryonated and unembryonated eggs was counted under a binocular stereo zoom microscope (Olympus SZ40, Japan).

$$\text{Percentage efficacy} = \frac{\text{Total number of eggs added} - \text{Number of larvae hatched}}{\text{Total number of eggs added}} \times 100$$

2.3.4 Larval Paralysis Assay (LPA)

The efficacy of aqueous leaf extracts of *A. indica* in LPA was determined by the proportion of the paralysed third-stage larva incubated at increasing concentrations of the extracts using Ivermectin as a standard positive control.

Briefly, 500 µL of an aqueous leaf extract of *A. indica* was added at final concentrations of 0.5%, 1%, 2% & 5% in each well of 24 multiwell plates. 500 µl of 0.9µg/ml concentration of Ivermectin was taken as a standard positive control, and water was taken as a negative control. 500µl of larval suspension containing approximately 30 larvae was added to each well. The mobile and immobile larvae were counted at 15-minute intervals for a 60-minute duration at room temperature. The paralysed larvae were counted and expressed as a percentage.

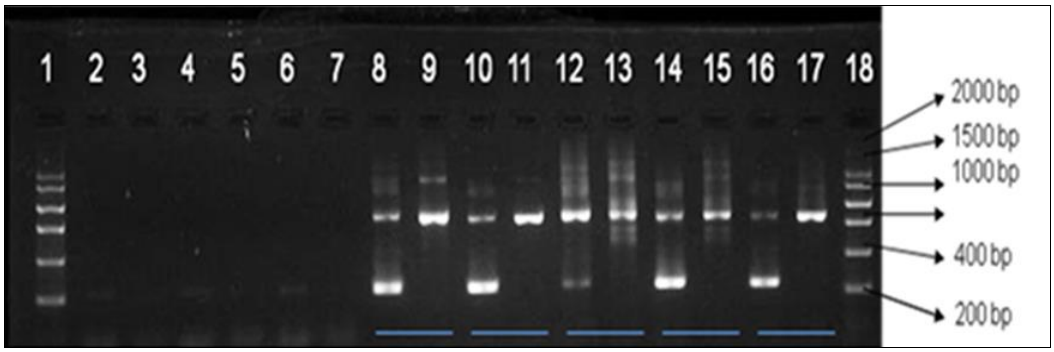
3. Results

3.1 Faecal egg count reduction test (FECRT)

The EPG on 0 and 14-day samples was calculated using McMaster, and the assessment of the development of resistance was calculated using RESO 4.0 software. FCERT was carried out in 560 sheep from organized sheep farms of Salem, Karur, Nagercoil, Melmaruvathur, Kancheepuram and Thiruvallur for the development of resistance to benzimidazole. A reduction of faecal egg count less than 95% or the lower 95% confidence limit less than 90% was considered as resistance, and if any of the above parameters were not met, then it was classified as suspected resistance. In this study, out of 560 samples tested, 106 samples from Kancheepuram and Thiruvallur districts showed resistance, and the remaining 454 samples were susceptible to Benzimidazole.

3.2 Allele-specific PCR (AS-PCR)

Allele-specific PCR was performed based on the method of Silvestre and Humbert (2000) [24]. In this resistant fragment size of 250 BP could be obtained (Figure 2). Development of resistance could be detected both by FECRT and also AS-PCR for samples from organized sheep farms of Kancheepuram and Thiruvallur districts.



Lane-8,10,12,14,16- 250bp- Resistance ; Lane- 9,13,15,17- 770 bp- β tubulin

Fig 2: AS-PCR showing amplification of resistant gene (rr)

3.3 Egg Hatch Assay

The egg hatch assay was used to test the efficacy of the aqueous leaf extract of *A. indica*. The results of the Egg Hatch Assay are presented in Table 1.

Table 1: Efficacy of Aqueous leaf extract of *A. indica* in EHA

EHA	Concentration of aqueous leaf extract of *A. indica*			
	0.5%	1%	2%	5%
Per cent inhibition of egg hatch	0	1.83±1.05[b]	22.08±1.50[c]	35.42±1.87[d]

[a,b] Mean values with different superscripts differ significantly (p<0.05%)

The results show that there was significant inhibition of egg hatch by different concentrations of aqueous leaf extract of *A. indica* after 48 hours of incubation. 5% aqueous leaf extract of *A. indica* showed 11.67 % inhibition of egg hatch.

3.4 Larval Paralysis Assay

The results of Larval Paralysis are presented in Table 2.

Table 2: Mean larval paralysis in aqueous leaf extract of *A. indica*

Time	PBS	Concentration of aqueous leaf extract of *A. indica*				Ivermectin (0.03 µg/ml)
		0.5%	1%	2%	5%	
15 Min	0	0	4.44±0.70[a]	7.22±1.02[a]	13.89±1.03[a]	83.89±5.24
30 Min	0	0	7.78±0.70[b]	11.67±1.14[b]	17.78±1.41[b]	98.33±2.87
45 Min	0	0	11.11±1.41[c]	18.89±1.11[c]	23.33±1.49[c]	100±0
60 Min	0	0	14.99±1.14[d]	19.45±1.02[c]	28.89±1.11[d]	100±0

Mean larval paralysis with different superscripts differs significantly (p<0.05%)

The LPA revealed that an increased percentage of paralysis occurred as the concentration of aqueous leaf extract of *A. indica* was increased from 0.5% to 5%.

4. Discussion

The FECRT is the *in vivo* test that determines the efficacy of an anthelmintic drug by comparing the EPG of faeces before and after treatment (Boersema, 1983; Presidente, 1985) [26-27]. The method described by Coles *et al*., 1992 [28] for FECRT was adopted in this study.

Benzimidazole drugs selectively bind to β-tubulins of the parasitic nematodes, resulting in inhibition of microtubule formation. The insoluble polymeric microtubules are formed from soluble alpha- and beta-tubulin molecules. The microtubules of nematode parasites play vital cell functions *viz.*, intracellular transport of nutrients, cell division, shaping and motility. Genetic analysis reports revealed that beta-tubulin polymorphism at codons 167 and 200 of the beta-tubulin isotype-1 led to changes in the amino acid sequence, resulting in the expression of tyrosine in the resistant nematodes instead of phenylalanine (Hoti *et al*., 2003) [29].

Prichard *et al*., 1980 [30] reported that the egg hatch assay is a sensitive test with repeatability, rapid and economical for testing anthelmintic resistance in a single nematode species. In this study, thiabendazole-resistant *H. contortus* was used to assess the anthelmintic potential of aqueous leaf extract of *A. indica*. The mean percentage of egg hatch in one hundred and six resistant samples was above the discriminating dose of 0.1 µg per ml of TBZ. Egg hatched even at higher concentration of TBZ i.e. 0. 5 µg per ml. This is in agreement with the report of Le Jambre (1976) [31]. An ED_{50} value in excess of 0.1 µg TBZ per ml in the egg hatch assay, indicative of benzimidazole resistance (Coles *et al*., 1992) [28]. Whereas, the susceptible eggs rarely hatched in TBZ solution at concentrations greater than 0.1 µg/ml (Taylor *et al*. (2002) [32]. Lourderaj (2005) [25] reported an ED_{50} value of 0.8 and 0.6 µg TBZ per ml indicating anthelmintic resistance of gastrointestinal nematodes of sheep in the regions of Erode and Othiawakam of Tamil Nadu. Easwaran *et al*. (2009) [33] reported ED_{50} values of TBZ at 0.627, 0.678 and 0.388 µg/ml of TBZ and also indicated that the nematode parasites of sheep in the southern districts of Tamil Nadu developed benzimidazole resistance. This study reports benzimidazole resistance in gastrointestinal parasites of northern districts of Tamil Nadu and in agreement with the earlier reports.

The leaf extracts of *A. indica* exerted an anthelmintic effect in a dose-dependent pattern in this study. Rehman *et al*. (2023) [18] reported only 1.12 and 3.57% egg hatching in 25mg/mL concentration of methanolic and ethyl acetate extracts of *A. indica*. *In vivo* studies (FECRT) revealed a significant reduction in egg counts in goats (Priscilla *et al*., 2014 [13]; Dongre *et al*., 2014) [10] and cattle (Jamra *et al*., 2015) [11] treated with *A. indica* leaf powder and leaf extracts.

Martin and LeJambre (1979) [34] developed the larval paralysis assay while investigating the resistance to levamisole and morantel. In LPA the percentage of paralysed third-stage larvae was counted after incubating them in a serially diluted anthelmintic solution for at least 60 min, and the dose-response line was plotted to compare the efficacy with a known anthelmintic. The present study revealed that aqueous leaf extract of *A. indica* has the potential anthelmintic property in worms that were resistant to benzimidazole. The *A. indica* leaves reported to have 20 phyto compounds based on the peaks identified by Gas chromatography-Mass Spectroscopy with a molecular weight ranging from 144 to 336 with higher levels of oleic acid, nanodecanoic acid, octadeconoic acid and arabinopyranoside (Adams *et al*., 2023) [20].

5. Conclusions

A total of 560 dung samples from organized sheep farms of Salem, Karur, Kanniyakumari, Kancheepuram and Thiruvallur districts of Tamil Nadu were examined by FECRT for the development of resistance to benzimidazole. The samples from Kancheepuram and Thiruvallur districts showed resistance to benzimidazole by FECRT. The allele-specific PCR (AS-PCR) also confirmed the benzimidazole resistance in samples from Kancheepuram and Thiruvallur districts. The aqueous leaf extracts of *A. indica* were tested against benzimidazole-resistant strongyle nematodes of sheep reared in these farms. Increased inhibition of egg hatch was noticed in 5% aqueous leaf extracts of *A. indica* (35.42±1.87%) compared to other extracts. The maximum mean larval paralysis observed was 28.89±1.11% in 5% aqueous extract at 60 min. It was observed that increased concentration of extracts resulted in increased efficacy of inhibition on egg hatch and mean larval paralysis. It is concluded that *A. indica* could be a promising phytomedicine for the benzimidazole-resistant nematodes of sheep. There is ample scope for further exploration of *A. indica* as an alternative phytomedicine to combat anthelmintic resistance in livestock.

Conflict of Interest: Not available

Financial Support: Not available

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