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EVALUATION OF ANTHELMINTIC POTENTIAL OF ETHANOLIC LEAF EXTRACT OF *Vitex negundo* Linn. AGAINST BENZIMIDAZOLE-RESISTANT HAEMONCHUS CONTORTUS OF SHEEP

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Abstract: This study aimed to evaluate the anthelmintic potential of ethanolic extract of *Vitex negundo* leaves against *Haemonchus contortus* 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 to elucidate the development of resistance to benzimidazole. The samples from Salem, Kancheepuram and Thiruvallur districts showed resistance to benzimidazole by FECRT. The Allele specific PCR (AS-PCR) confirmed resistance to benzimidazole in samples from Salem, Kancheepuram and Thiruvallur districts. Ethanolic extracts of *Vitex negundo* leaves at different concentrations *viz.,* 0.5 1,2 and 5 per cent were tested against *H. contortus* of sheep reared in these farms with BZ resistance. Maximum inhibition of egg hatch was noticed in 5% ethonolic extracts of *V. negundo* (5.00±0.91%) compared to other concentrations. The larval paralysis assay (LPA) revealed that an increased percentage of paralysis occurred as the concentration of extract and duration of exposure were increased. The maximum LPA of 7.23±0.55% was observed in 5% ethanolic concentration of *V, negundo* at 60 min. It was observed that increased concentration of extracts resulted in increased efficacy of inhibition on egg hatch. It is concluded that *V. negundo* leaves could be a potential phytomedicine for the benzimidazole-resistant nematodes of sheep.
Keywords: Sheep, Nochi, *Vitex negundo*, ethanolic leaf extracts, Benzimidazole, anthelmintic resistance.

INTRODUCTION

The anthelmintic resistance poses a serious threat in the sheep industry in terms of reduced performance and failure to control clinical disease on a global basis. 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). Under intensive systems of animal management, most helminth control strategies currently rely heavily on the use of anthelmintics. However, the regular use of any chemical to control infectious organisms poses the risk of developing resistant populations. World-wide reports of nematodes with resistance to one or more of the available broad-

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spectrum anthelmintic groups are therefore of great concern to all of those involved in advising on parasite control. To maintain the production benefits of intensive livestock production systems, the efficacy of available compounds must be maintained so that worm control measures remain effective. When all the available compounds have been exhausted, there are at least chances of finding alternate compounds in the form of botanical or herbal anthelmintics in India due to the vast biodiversity of flora. 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)

Vitex negundo (*Nochi,* Tamil; *Nirkundi,* Hindi; Five-leaved chaste tree, English) is a large aromatic shrub, sometimes a small slender tree, found throughout the greater parts of India. Traditionally, leaves are reported to possess a tranquillising effect, insecticidal properties and are laid over grain to ward off insects (Venkateshwaralu, 2012; Ladda and Magdum, 2012; Khan *et al*., 2021). The leaves of *V. negundo are* reported to be rich in terpenoids, flavonoids, iridoids and glycosides (Venkatesswaralu, 2012; Khan *et al.,* 2021). This paper reports the anthelmintic potential of the ethanolic extracts of *V. negundo* leaves for combating benzimidazole-resistant *H. contortus* of sheep in Tamil Nadu.

MATERIALS AND METHODS

Collection and processing of the plant

Vitex negundo 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 (Fig. 1a – 1c)

Preparation of extract

Four hundred grams of powder of *V. negundo* leaves were macerated using one litre of ethanol 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. The yield of the ethanolic extract of *V. negundo* leaves was 1 per cent. The dried ethanolic extract was stored at -20 °C until further use.

		
Fig 1a. *Vitex negundo* plant leaves	Fig 1b. Dried *V. negundo* leaf powder	Fig 1c. Ethanolic extraction of *V. negundo* leaf powder

Detection of Anthelmintic Resistance

Anthelmintic resistance was detected by using *in vivo* faecal egg count reduction test, allele-specific polymerase chain reaction, *in vitro* Egg hatch assay and larval paralysis assay.

Faecal Egg Count Reduction Test (FECRT):

This is the most common *in vivo* test to detect anthelmintic resistance. This test provides an estimation of anthelmintic efficacy by comparing faecal egg counts of animals before and after treatment.

Dung samples from sheep farms from Karur, Melmaruvathur, Nagercoil, Salem, Kancheepuram and Thiruvallur treated with benzimidazoles 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. The dung samples were transported to the laboratory within 48 hrs at 4°C and subjected to Egg Per Gram (EPG), egg hatch assay and larval paralysis assay.

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 filtrate was collected and centrifuged at 2000 rpm for 2 minutes. The supernatant was poured off. 26 ml of saturated salt solution was added to the sediment and agitated. This suspension was loaded into a three-chambered McMaster slide chamber. The samples from different places were loaded separately in each chamber of Mc Master Slide. EPG was calculated by multiplying the number of eggs counted by 25.

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°C for 3 min, followed by 36 cycles (denaturation 94°C for 55 sec, annealing 57°C for 55 sec and extension 72°C for 55 sec) with the final extension at 72°C for 10 min and the PCR products were visualized after gel electrophoresis using Gel Doc system.

Nested PCR was performed using amplicons of β tubulin 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)

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.

Analysis of PCR products

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 transparent submarine gel electrophoresis system (Broviga) and wells were prepared by placing 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 PCR products were visualized and photographed using a video gel documentation system (BIO RAD Gel Doc XR+).

Assessment of resistance by egg hatch assay (EHA)

The egg hatch assay method is an *in vitro* diagnostic method of Anthelmintic Resistance. This provides an accurate method for assessing the susceptibility of mixed nematode populations also and it is a more rapid and economic test. This test is based on the determination of the proportion of eggs that fail to hatch in solutions of increasing drug concentrations in relation to control wells.

Preparation of ethanolic *V. negundo* extracts: Ethanolic extracts of *V. negundo* were dissolved in dimethyl sulfoxide (DMSO) to prepare the final concentration of 0.5%, 1%, 2% and 5% in the 24 multiwell plate.

Preparation of Thiabendazole (TBZ) stock and working solutions: Pure thiabendazole Sigma-T-8904) 50mg was transferred into a 50 ml beaker, and 50ml of DMSO was added and mixed thoroughly to prepare a dimethyl sulfoxide stock solution of 1000 ppm of thiabendazole. Using the stock solution, a suitable range of working solutions was prepared to a final concentration of 0.1, 0.2, 0.3 µg/ml.

The method described by Lourderaj (2012) was followed for the egg hatch assay with minor modifications. Briefly, to each well of a 24 multiwell plate, 500µl of egg suspension containing approximately 40 eggs was added. 500 µl of working solution of 0.1, 0.2, 0.3, µg/ml TBZ was added to each benzimidazole control wells. 500µl of ethanolic *V. negundo* extract was added with a final concentration of 0.5%, 1%, 2% & 5% in different wells. 500 µl of DMSO (99 per cent) was added to the negative control wells. The tests were carried out with six 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 25^0 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 under a binocular stereo zoom microscope (Olympus SZ40, Japan) and percentage of hatch was derived using the following formula to determine the resistance status. The percentage hatch was taken as a percentage of resistance as the larvae survived anthelmintic treatment.

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

After 48 hrs, the number of eggs embryonated and unembryonated was counted under a binocular stereo zoom microscope (Olympus SZ40, Japan). The percentage of unembryonated eggs was calculated to determine the susceptibility to the extracts used.

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

Thus, the percentage efficacy of ethanolic *V. negundo* extracts was assessed to tackle resistance.

Larval Paralysis Assay (LPA):

This assay discriminates between resistant and susceptible strains of parasites by estimating the proportion of third-stage larvae in tonic paralysis after incubation with increasing drug concentrations. To test the effect of ethanolic extracts of *V. negundo* leaves on larval paralysis, this assay was performed using Ivermectin as a standard positive control.

Briefly, in a 24-well multiplate 500 µL of an ethanolic leaf extract of *V. negundo* was added at final concentrations of 0.5%, 1%, 2% & 5% in each well. 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 tests were carried out with six replicates for each drug concentration and controls. The mobile and immobile larval counts were taken at 15-minute intervals for a 60-minute duration at room temperature. Paralysed larvae were counted and expressed as a percentage.

RESULTS AND DISCUSSION

Faecal egg count reduction test (FECRT)

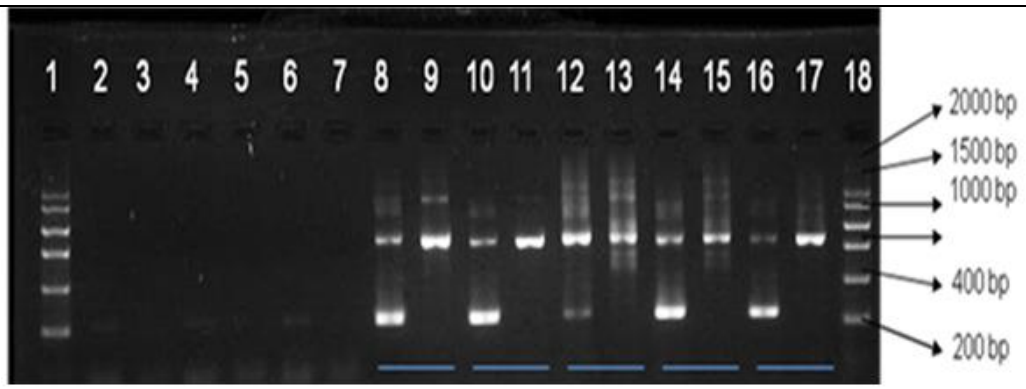
The FECRT provides an estimation of anthelmintic efficacy by comparing faecal egg counts of animals before and after treatment (Presidente, 1985; Boersema *et al.,* 1987). Guidelines that give precise details and recommendations on the use of the FECRT have been used in this study (Coles e*t al*., 1992).

EPG on 0 and 14-day samples was calculated using McMaster, and 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 was 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.

Allele – specific PCR (AS-PCR)

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



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

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

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, like cell division, shaping, motility and intracellular substrate transport. Genetic analysis reports revealed that beta-tubulin polymorphism at codon 167 and 200 of the beta-tubulin isotype-1 led to changes in the amino acid sequence which resulting in the expression of tyrosine in resistant worms instead of phenylalanine in susceptible worms, where benzimidazole binds between the beta-tubulin phenylalanine 167 and 200 residues.

Egg Hatch Assay for the detection of anthelmintic resistance

The egg hatch assay was used to test the efficacy of the ethanolic *V. negundo* extracts. The results of the egg hatch assay are presented in Table 1

Table.1 Efficacy of Ethanolic extract of *V. negundo* in EHA

EHA	Concentration of ethanolic extract *of V. negundo*			
	0.5%	1%	2%	5%
Per cent inhibition	0	0	1.67±0.83[a]	5.00±0.91[b]

[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 ethanolic extracts of *V. negundo* after 48 hours of incubation. 5% ethanolic *V. negundo* extract showed 5.00±0.91 inhibition of egg hatch.

Egg hatch assay was used to assess the resistance to thiabendazole in this study. The egg hatch assay is fast, inexpensive, sensitive and repeatable when a single species of nematode is involved in resistance (Prichard *et al.,* 1980). In this study, the assessment of resistance to thiabendazole in *H. contortus* was done. 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 and larvae hatched even at higher concentration of TBZ, i.e 0.3 µg per ml of TBZ. This is similar to the report of Le Jambre (1976), who showed that TBZ-resistant strains hatched in higher concentrations of TBZ than non-resistant strains. Coles *et al.* (1992) reported for the first time that eggs with an ED_{50} value in excess of 0.1 µg TBZ per ml were indicative of benzimidazole resistance. Taylor *et al.* (2002) reported eggs from susceptible individuals rarely hatched at concentrations greater than 0.1µg / ml of thiabendazole.

In Tamil Nadu, Lourderaj (2005) reported the development of resistance in worm population of Erode and Othiawakam with the ED_{50} values of 0.8 and 0.6 µg TBZ per ml. Easwaran *et al*. (2009) detected benzimidazole resistance in gastrointestinal nematodes of sheep in three farms of southern India with the ED_{50} values of 0.627, 0.678 and 0.388 µg / ml of TBZ. Assessment of resistance by EHA in this study was on similar lines and in agreement with the earlier reports from Tamil Nadu.

Larval Paralysis Assay

The results of Larval Paralysis are presented in Table.2

Table.2. Mean larval paralysis in Ethanolic *V. negundo* Extract

Time	PBS	Concentration of Ethanolic *V. negundo* Extract				Ivermectin (0.03 µg/ml)
		0.5%	1%	2%	5%	
15 Min	0	0	0	0	3.88±1.03[a]	83.89±5.24
30 Min	0	0	0	1.11±0.70[a]	4.44±0.70[a]	98.33±2.87
45 Min	0	0	0.55±0.55[a]	3.33±0.86[a]	7.22±1.02[b]	100±0
60 Min	0	0	4.99±1.14[b]	3.33±0.86[a]	7.23±0.55[b]	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 extract was increased from 0.5% to 5%. Larval paralysis test was developed for the detection of levamisole and morantel resistance (Martin and LeJambre, 1979). In the assay, infective

third-stage larvae are incubated for one hour in serial dilutions of the anthelmintic. After this time the percentage of paralysed larvae is determined at each concentration and a dose-response line plotted and compared to known anthelmintic. This study revealed that *V. negundo* extract has the potential anthelmintic property in worms that were resistant to benzimidazole as the leaves reported to have higher levels of total phenol, flavonoid and antioxidant activities (Khan *et al*., 2024)

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 Salem, Kancheepuram and Thiruvallur districts showed resistance to benzimidazole by FECRT. The allele-specific PCR (AS-PCR), resistance was confirmed in samples from Salem, Kancheepuram and Thiruvallur districts. Ethanolic *V. negundo* extracts were tested against benzimidazole-resistant strongyle nematodes of sheep reared in these farms. Increased inhibition of egg hatch was noticed in 5% ethanolic extracts of *V. negundo* (5.00±0.91%) compared to other extracts. It was observed that increased concentration of extracts resulted in increased efficacy of inhibition on egg hatch. The LPA revealed that an increased percentage of paralysis occurred as the concentration of extract and duration of exposure were increased. The maximum LPA of 7.23±0.55% was observed in 5% ethanolic concentration of *V, negundo* at 60 min. It is concluded that *V. negundo* could be a promising phytomedicine for the benzimidazole-resistant nematodes of sheep. There is ample scope for further exploration of *Vitex negundo* as an alternative phytomedicine to combat anthelmintic resistance in sheep.

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