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In-vitro Study on Acaricidal Efficacy of *Azadirachta indica* (Neem) Acetonic Extracts against *Rhipicephalus* (*B.*) *microplus* in Udaipur (Rajasthan)

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ABSTRACT: The present research was done to evaluate the acaricidal property of the acetonic extracts of seed, leaves and bark *Azadirachta indica* against larvae and adult stages of *Rhipicephalus* (*B.*) *microplus* ticks. The ticks and their eggs were treated with various concentrations of herbal acaricide (70, 50, 25 and 10 mg/ml). One control group with twice replications for each dilutions were used for the Adult Immersion Test (AIT) and Larval Packet Test (LPT). The seeds, leaves and bark of acetonic extracts of neem exhibited lethal effects on larval mortality which at 70 mg/ml concentration was 76%, 59.5% and 47% respectively. The treated *Rhipicephalus* (*B.*) *microplus* with acetonic extract of *A. indica* seeds, leaves and bark showed inhibition of oviposition (IO%) which was 77.3%, 50.13% and 49.96% respectively at 70 mg/ml concentration used *in vitro*. In acetonic extracts of seeds, leaves and bark, reproductive index (0.10), (0.22) and (0.22) respectively were observed at 70 mg/ml concentration. Among the three extracts of *A. indica* evaluated by AIT, the decrease in reproductive index and increase in the percent inhibition of oviposition, was evident during the study. The results indicated that these plant extracts have potential to be developed as herbal acaricides. *A. indica* has a great potential for the integrated control of ticks in animals due to its acaricidal efficacy and eco-friendly nature.

Keywords: Acaricidal potential, Acetonic extracts, *Rhipicephalus microplus*, Adult Immersion Test (AIT) and Larval Packet Test (LPT).

INTRODUCTION

Livestock rearing is one of the most important economic activities in the rural areas of the country contributing significantly to the national economy. Around 70 percent of Indian population is thriving on agriculture and allied sectors like livestock industry. The total livestock population is about 535.78 million in country with increase of 4.6 percent over Livestock census 2019. Today, most of the emerging infectious diseases (Zoonotic pathogens) and important haemo-protozoan diseases are transmitted by ticks (Jongejan and Uilenberg 2004). The damage caused by ticks and tick born diseases (TTBDs) to livestock is considered very high (Ghosh *et al.*, 2006). The global loss due to ticks and tick borne diseases (TTBDs) was estimated to be between US$ 109 billion annually (Ghosh *et al.*, 2014) while in India the cost of controlling TTBDs has been estimated as US$ 498.7 million/annum (Minjauw *et al.*, 2003). The resistance of the tick *Rhipicephalus* (*B.*) *microplus* to synthetic acaricides gave rise to the need for new scientific research on other ways to control this tick. For thousands of years, humans have been using herbal remedies to cure and prevent various illnesses. Out of many herbal products, neem has numerous properties which have promoted its use in both the medical and veterinary fields. Acetonic seed, leaves and bark extracts of *Azadirachta indica* (Meliaceae) have medicinal properties used to control skin infections and applied as insect repellant and sometimes used as pesticide (Schmutterer, 1990; Sofowora, 1993; Isman, 1997). US National Academy of Sciences recognized the importance of neem tree in 1992 and entitled neem as "a tree for solving global problem's" (Singh *et al.*, 2017).

MATERIALS AND METHODS

A. Preparation of plant extract

The seeds, leaves and bark of *A. indica* were collected from fields, road side and gardens in and around Vallabhnagar tehsil of Udaipur (Rajasthan). The plant materials were dried in shade at room temperature and subsequently pounded in to powder using pestle and

mortar or with an electronic blender. Further, to remove the ungrounded parts of the material, the powder was sieved out with the help of muslin cloth. After that the powders were stored in well labelled air tight containers and kept in a cool place (Shyma *et al.*, 2014). Preparation of Plant Extracts was done by using organic solvent of acetone. Plant material was dissolved in solvent and macerated for two days. After that the mixture was filtered out using Whatman No. 1 filter paper. The filtrate was kept in water bath for evaporation at 45°C and then dried at room temperature. The dried extracts were stored properly in sealed beakers in refrigerator at 4°C and used as and when required. Different concentrations (10, 25, 50 and 70 mg/ml) of each extract residues were prepared in respective solvent acetone. Controls were run side by side for all solvents used in research.

B. Collection and identification of ticks

Ticks were collected in small boxes with a few small holes allowing air to circulate and brought to the Department of Veterinary Parasitology, C.V.A.S. Navania, Vallabhnagar, Udaipur (Rajasthan) for morphological identification of ticks according to keys of Soulsby (1982).

(1) Larval Packet Test (LPT). The larval packet test (LPT) was conducted according to FAO (2004) guidelines with minor modifications. After identification the ticks were washed with distilled water and dried with filter paper. Ticks were then placed in incubator at 27 °C and relative humidity of 85% for laying of eggs. Cattle ticks were examined daily until oviposition. The eggs were separated and allowed to hatch in glass vials with cotton plug and kept in optimal conditions. The obtained egg ticks were maintained at 27.0 ±1.0ºC and 85-92% relative humidity for 21 days in desiccators placed in BOD incubator. The larvae aged 21 days were subjected to larval packet test. For this test Whatman filter paper NO. 1 in parallelogram shape (12 cm × 18 cm) was impregnated with different dilutions (70, 50, 25 and 10 mg/ml) and one control group for each concentration of test. Then the packet was dried for about half an hour at room temperature. Treated papers were folded into packets and a fixed number of 100 larvae were placed. The packets after placing in dessicator were kept in BOD incubator for the 24 hours and total mortality of larvae was counted using the following formulae:

$$\text{Percent mortality} = \frac{\text{Number of dead larvae}}{\text{Total number of larvae}} \times 100$$

(2) Adult Immersion test (AIT)

The ticks were collected from naturally infested cattle. The obtained ticks were divided into groups. Ten engorged females were placed in each treatment group and one control group for each concentration of test. These ticks were weighed to make homogeneous group for study. They were immersed for 5 min in acetonic extracts of *A. indica* in different concentrations (70, 50, 25 and 10 mg/ml) and one control group was used in the bioassay. Ticks were removed and left for drying on filter paper and transferred to the petridish. The petridish was then placed in dessicator. Dessicator was placed in BOD incubator for the 15 days these ticks were observed for inhibition of oviposition and reproductive index (Drummond *et al.*, 1983).

$$\text{Reproductive index} = \frac{\text{Weight of egg laid (mg)}}{\text{Weight of adult females (mg)}}$$

$$\text{Inhibition of oviposition (IO \%)} = \frac{\text{RI (control group)} - \text{RI (treated group)}}{\text{RI (control group)}} \times 100$$

D. Statistical analysis

Statistical analysis for significance of difference in occurrence among various occurrence and clinical data will be done as described by Snedecor and Cochran (1994). The data will be analyzed by using statistical software program.

RESULTS AND DISCUSSION

Rhipicephalus (B.) microplus was morphologically identified in C.V.A.S, Udaipur (Rajasthan). For testing percentage larval mortality, inhibition of oviposition (IO %) and reproductive index were studied at four treatment groups (70, 50, 25 and 10 mg/ml) and one control group was used in acetonic extracts of seeds, leaves and bark of *A. indica* for the bioassay. Larvae of *Rhipicephalus (B.) microplus* were exposed to filter paper envelopes impregnated with different concentrations of neem. Four envelopes were impregnated with each tested solution. 100 larvae of 21 days old were fasted and placed in each envelope. Larval mortality was observed 24 h after treatment in LPT test. Then Acaricidal efficacy of plant extracts was estimated by using Adult Immersion Test (AIT).

A. Efficacy in acetonic extracts of A. indica in Larval Packet Test (LPT)

The different dilutions of acetonic extracts of seeds, leaves and bark of *A. indica* varied from 10 to 70 mg/ml, where peak mortality was observed from seeds (76%) followed by leaves (59.5%) and bark (47%) respectively at a concentration of 70 mg/ml. A total of four treatment groups and one control group were used in experiment. In control group no mortality of ticks was observed. A significant larval mortality was produced by application of extracts of 50 mg/ml, 25 mg/ml and 10 mg/ml where seeds (53.5%, 27.5% and 12%), followed by leaves (43.5%, 22.5% and 7.5%) and bark (35.5%, 18% and 7%) respectively. With the increase in concentration level the percent mortality rate also increased, as shown in (Table 1).

Table 1: The results of larval packet test (LPT) ofacetonic extracts of seed, leaves and bark of *A. indica* on percent mortality against *R.* (B.) *microplus.*

Product	Concentration of extract (mg/ml)	Live larvae	SE	Dead larvae	SE	% of Larval mortality	SE
A. indica (Seed) extracts	Control	100^{e}	0	0^{a}	0	0^{a}	0
	70	24^{a}	2	76^{e}	2	76^{e}	2
	50	46.5^{b}	1.5	53.5^{d}	1.5	53.5^{d}	1.5
	25	72.5^{c}	0.5	27.5^{c}	0.5	27.5^{c}	0.5
	10	88^{d}	1	12^{b}	1	12^{b}	1
A. indica (leaves) Extracts	Control	100^{e}	0	0^{a}	0	0^{a}	0
	70	40.5^{a}	0.5	59.5^{e}	0.5	59.5^{e}	0.5
	50	56.5^{b}	0.5	43.5^{d}	0.5	43.5^{d}	0.5
	25	77.5^{c}	0.5	22.5^{c}	0.5	22.5^{c}	0.5
	10	92.5^{d}	0.5	7.5^{b}	0.5	7.5^{b}	0.5
A. indica (bark) Extracts	Control	100^{e}	0	0^{a}	0	0^{a}	0
	70	53^{a}	1	47^{e}	1	47^{e}	1
	50	64.5^{b}	1.5	35.5^{d}	1.5	35.5^{d}	1.5
	25	82^{c}	1	18^{c}	1	18^{c}	1
	10	93^{d}	2	7^{b}	2	7^{b}	2

Means bearing different superscript in the same column differ significantly P<.05

In LPT test after 24 hours of contact, the mortality rate varied according to the concentrations and the acetonic extracts showed highest larvicidal effect. Acetonic seed extracts showed higher mortality (76%, 53.5%, 27.5% and 12%), followed by leaves (59.5%, 43.5%, 22.5% and 7.5%) and bark (47%, 35.5%, 18% and 7%) respectively at 70, 50, 25 and 10 mg/ml concentration where as control groups showed no mortality of ticks larvae under evaluation. The results of the present study were in accordance with Choudhury *et al*. (2009) who reported that the neem seed oil tested against the larvae of *Boophilus decoloratus* ticks showed 100 percent mortality and Nithya *et al*. (2017) who reported that extracts of *A. indica* showed maximum mortality rate of ticks over *Calotropis procera* when tested individually. Micheletti *et al.* (2009) reported a mortality of 65% on use of neem leaves.

B. Efficacy of acetonic extracts of seed, leaves and bark of A. indica against R. (B.) microplus in Adult Immersion Test (IO%)

The percent adult tick inhibition of oviposition (IO%) and reproductive index produced by the crude acetonic extracts of seed, leaves and bark of *A. indica* against *R. (B.) microplus* varied in different concentrations, ranging from 10 to 70 mg/ml. A significant percentage inhibition of oviposition (IO%) was observed at 70, 50, 25 and 10 mg/ml concentrations of the extracts of seed, leaves and bark which were (77.3%, 68.34%, 60.70%, 42.09%) in seed extracts, (50.13%, 37.26%, 22.9% and 10.84%) in leaf extracts and (49.96%, 34.88%, 17.5% and 9.2%) in bark extracts, respectively. RI at 70 to 10 mg/ml concentrations of acetonic seed extract showed minimum reproductive index (0.10, 0.14, 0.18 and 0.26) whereas leaves and bark showed reproductive index (0.22, 0.28. 0.35 and 0.40) and (0.22, 0.29. 0.37, 0.41) respectively. In AIT the dependent decrease in reproductive index and increase in inhibition of oviposition was observed from concentration 10 to 70 mg/ml. No mortality of ticks was observed in control group, as shown in Table 2.

Table 2: Acaricidal efficacy of different concentrations acetonic extracts of *Azadirachta indica* (seed, leaves and bark) on *R. (B.) microplus.*

Product	Conc. of extract (mg/ml)	Live ticks weight (gm)Mean)	(SE)	Weight of eggs laid (gm) (mean)	(SE)	Reproduction Index (RI) (Mean)	(SE)	%IO (Mean)	(SE)
A. indica (seed) extracts	Control	0.760	0.040	0.355^{d}	0.025	0.46^{c}	0.01	0^{a}	0.00
	70	0.560	0.07	0.06^{a}	0.01	0.105^{a}	0.03	77.3^{c}	8.42
	50	0.550	0.08	0.07^{ab}	0.01	0.14^{ab}	0.02	68.34^{bc}	6.12
	25	0.505	0.04	0.085^{b}	0.01	0.18^{b}	0.03	60.70^{bc}	7.3
	10	0.525	0.04	0.14^{c}	0.02	0.265^{c}	0.01	42.09^{b}	4.32
A. indica (leaves) Extracts	Control	0.760	0.04	0.355^{d}	0.025	0.46^{c}	0.01	0^{a}	0.00
	70	0.88	0.01	0.202	0.00	0.22^{a}	0.01	50.13^{d}	1.20
	50	0.79	0.03	0.231	0.04	0.28^{ab}	0.05	37.26^{cd}	9.61
	25	0.81	0.08	0.294	0.04	0.355^{bc}	0.02	22.9^{c}	3.76
	10	0.85	0.01	0.349	0.01	0.409^{bc}	0.01	10.84^{b}	6.18
A. indica (Bark) Extracts	Control	0.760	0.04	0.355^{d}	0.025	0.46^{c}	0.01	0^{a}	0.00
	70	0.70	0.02	0.167^{a}	0.003	0.229^{a}	0.002	49.96^{e}	1.73
	50	0.71	0.03	0.214^{b}	0.014	0.299^{b}	0.005	34.88^{d}	0.22
	25	0.65	0.50	0.249^{b}	0.019	0.379^{c}	0.009	17.5^{c}	0.27
	10	0.71	0.01	0.29^{bc}	0.002	0.417^{cd}	0.00	9.2^{b}	1.43

Means bearing different superscript in the same column differ significantly P<.05

Among the different concentrations of acaricidal efficacy of acetonic extracts of seeds, leaves and bark extracts of neem**,** the highest inhibition of oviposition was observed in seeds (77.3%, 68.34%, 60.70% and 42.09%) followed by leaves (50.13%, 37.26%, 22.9% and 10.84%) and bark (49.96%, 34.88%, 17.5% and 9.2%) respectively at the 10 to 70 mg/ml dilutions. Concentrations of acetonic seed extracts showed minimum reproductive index (0.10, 0.14, 0.18 and 0.26) whereas leaves and bark showed reproductive index (0.22, 0.28. 0.35 and 0.40) and (0.22, 0.29. 0.37, 0.41) respectively. In AIT a dependent decrease in reproductive index and increase in inhibition of oviposition was observed. The results of the present study were similar to Das *et al.* (2015) who evaluated that the efficacy of neem oil and reported 56.25% against tick infestation in goats. Sanjib (2007) stated that the efficacy of neem oil was 70% against tick infestation in goats. Kalakumar *et al.* (2000) reported that neem oil was found 60.75% effective in cattle infested with ticks. Srivastava *et al.* (2008) observed that out of eight plants extract, *A. indica* seed extract was most effective (80%) after 5 h of treatment.

Among the different extrascts tested, different concentration of neem seed extracts was found to be most effective. The larvicidal and acaricidal effect of Neem seed were probably due to azadirachtin, triterpenoides, salannin and nimbin. Higher concentration of abundant azadirachtin, a triterpene to which most of the anti arthropod activity of this plant is attributed. Azadirachtin is associated with blocking the synthesis and release of moulting hormones (ecdysteroids) from the prothoracic gland, leading to incomplete ecdysis in immature insects. In adult female insects, a similar mechanism of action leads to sterility (Isman, 2006).

CONCLUSION

The results indicated that the plant extracts have potential to be developed as herbal acaricides. Acetone extracts prepared from the seed, leaves and bark of *A. indica* exhibited excellrnt acaricidal acativity against the tick's species. Further studies, especially *in-vitro* evaluation needs to be conducted as well as the isolation and identification of the compounds responsible for the acaricidal activity in these extracts. This will be beneficial for discovery and development of noval natural acaricides.

FUTURE SCOPE

These conventionally used acaricidal and larvicidal drugs are responsible for drug resistance after prolonged use. In the past years, research studies on plants have been carried out to prospect bioactive molecules with acaricidal properties because they are rapidly degraded, decrease poisoning of human applicators and non-target organisms, reduce environmental contamination, and decrease the development of resistance to these substances. The herbal acaricdes also provide cost effective alternative to chemical acaricidals. Several herbal agents are well known for their larvicidal, acaricidal potency and herbal eco-friendly nature and Neem is one of them.

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Conflict of Interest. None.

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