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In-vitro study on Acaricidal efficacy of *Azadirachta indica* (Neem) aqueous extracts against *Rhipicephalus* (B.) *microplus* in Udaipur (Rajasthan)

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Abstract
The objectives of this study were to evaluate the Acaricidal activity of the aqueous extracts of *Azadirachta indica* (seed, leaves and bark) against cattle tick, *Rhipicephalus* (*Boophilus*) *microplus* in Udaipur (Rajasthan) with various morphological details of cattle ticks. The Percentage larval mortality, inhibition of oviposition (IO%) and reproductive index were studied at various serial concentrations (70%, 50%, 25% and 10%) and one control group with twice replications for each concentration were used in the bioassay. The larval packet test was performed parallel to the adult immersion test for engorged female ticks for three aqueous extracts of *A. indica.* An aqueous seed, leaves and bark extract of neem was showed mortality of 54%, 56.5% and 36% respectively at 70% concentration LPT after 24 hrs. of treatment. Tick species and seed, leaves and bark extract concentration had significant effect on tick mortality ($P< 0.05$). As per results for AIT showed aqueous seeds, leaves and bark extract of *A. indica* at 70% concentration was IO% 54.27%, 62.05% and 36.57% respectively. In aqueous extracts of seeds, leaves and bark reproductive index (0.21), (0.17) and (0.29) were observed at 70 mg/ml concentration among the three extracts of *A. indica* evaluated.

Keywords: Acaricidal, aqueous, *Azadirachta indica*, *Rhipicephalus* (B.) *microplus*

Introduction
The cattle tick *Rhipicephalus* (Boophilus) *microplus* is one of the main responsible for economic losses to production in tropical and subtropical countries. The impact of ticks in the cattle industry is attributed to a combination of direct and indirect effects. Direct effects include skin damage from tick bites, blood loss and toxicity from the bites, reduced animal weight gain and reduced milk production. It is also estimated that a single female *R. (B.) microplus* tick is responsible for the loss of 1.18 ± 0.21g body weight in crossbred cattle (Jonsson, 2006) [5]. Indirect effects are related to the transmission of tick borne haemoprotozoan diseases (Sharma *et al.*, 2012, Mondal *et al.* 2013) [6, 11] observed that a single tick consumes a minimum of 30 drops of blood for completion of its life cycle and hence the most important adverse effects of tick infestation in animals are the anemia and retardation of growth. Historically, the method further explored against the cattle tick has been the traditional administration of chemical compounds resistance have been developed in ticks (Willadsen & Kemp, 1988) based on the use of active principles that act in various life stages of the parasite and its vectors. The indiscriminate use of acaricide compounds has, however, culminated in the appearance of populations of *R. (B.) microplus* resistant to these products, generating large expenses due to ineffective treatments. Plant extracts used in ethno-veterinary medicine represent a cheaper and easily accessible medicine for tick control (Nchu *et al.*, 2005) [9]. These plants like *Azadirachta indica* were taken into consideration during this study. The ingredients of plants and herbs are known to possess insecticidal, growth inhibiting, anti-molting and repellent activities (Habeeb, 2010) [4].

Material and Methods
Plant collection, identification, preparation of aqueous extracts
Seeds, leaves and bark of *Azadirachta indica* were collected and air dried at room temperature and later grinded and sieved through a mesh (2 mm size) and kept powder tight zip poly bag until processed. Powder of neem seeds, leaves and bark were dissolved in clean tap water. The mixtures were kept for 2 days in tightly sealed vessels at room temperature and filtered through muslin cloth. The extracted liquid was subjected to water bath evaporation at 45-50 °C

to remove the solvent. The semi-solid extract was kept under a ceiling fan to dry and kept in a refrigerator at a temperature of 0-4 °C until its use. Four serial concentrations (70%, 50%, 25% and 10%) and one control group with twice replications for each concentration were used in the bioassay.

Collection and identification of ticks and production of larva

The ticks were collected and preserved in 70% alcohol in clean, well-stopper glass vials which labeled properly and transferred to the laboratory and identified according to taxonomic keys of Walker *et al*. (2003) [13].

Test procedures

Adult immersion test (AIT)

Engorged Ten females' ticks from the field was washed with distilled water and kept for drying on filter paper and weighed. The ticks were selected for each group and immersed for 5 min in different concentration diluted extracts. The control group was immersed in the same volume of distilled water. After immersion, the ticks were placed in separate Petri dishes. The Petri dishes were placed in an incubator at 85±2% relative humidity. These ticks were observed for oviposition and reproductive index. The percent adult tick mortality and the weight of the eggs laid by the treated ticks were recorded in comparison with the control. The index of egg laying and percentage inhibition of fecundity was calculated using formulae.

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

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

Larval packet test (LPT)

The female ticks were washed, placed in sterile Petri dishes and incubated in a BOD chamber at 27±1 ^{0}C and relative humidity above 90% to promote oviposition. Seed ticks placed in the desiccators' under the same temperature and humidity conditions to obtain the larvae. About 100 larvae were used in each test, with ages between 14 and 21 days. They were placed between pieces of filter paper (2 cm × 2 cm) previously moistened with the solutions prepared, at the same concentrations described for the assays with the engorged females, and then closed to form packets, with twice repetitions for each concentration. Next, they were opened, and the live and dead number of larvae was counted Fernandez *et al.*, (18) [3].

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

$$\text{Corrected percent mortality} = \frac{\%\ \text{Test mortality} - \%\ \text{Control mortality}}{100 - \%\ \text{Control mortality}}$$

Fig 1: Larave hatching in glass vials Counting dead and live larve

Statistical analysis

The data generated were analyzed by statistical analysis and groups were compared using one-way ANOVA for repeated measurements using SPSS Software version 20. A value of $p<0.05$ was considered as significant (Snedecor and Cochran, 1980) [12].

Fig 2: *Rhipicephalus (Boophilus) microplus*

Results

Efficacy of aqueous extracts of *Azadirachta indica* (Seed, leaves and bark) in larval packet test (LPT)

The concentrations of aqueous extracts of *A. indica* (seed, leaves and bark) of varied from 10 to 70 mg/ml. The peak mortality was observed in seed (54%) followed by leaves (56.5%) and bark (36%) at 70 mg/ml concentration among the three extracts of *A. indica* under evaluation. A total of four treatment groups and one control group were used in experiment. A significant larval mortality produced by application of extracts of 50 mg/ml, 25 mg/ml and 10 mg/ml were seed (38.5%, 21% and 9%), leaves (40%, 21% and 7.5%) and bark (25.5%, 14% and 5%) respectively. With the increase in concentration level the percent mortality rate also increased as shown in (Table. 1).

Table 1: The results larval packet test (LPT) to aqueous extracts *Azadirachta indica* (Seed, leaves and bark) 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^d	0	0a	0	0a	0
	70	46^a	1	54^e	1	54^e	1
	50	61.5^b	0.5	38.5^d	0.5	38.5^d	0.5
	25	79^c	1	21^c	1	21^c	1
	10	91^d	1	9b	1	9b	1
A. indica (Leaves) Extracts	Control	100^e	0	0a	0	0a	0
	70	43.5^a	0.5	56.5^e	0.5	56.5^e	0.5
	50	60^b	1	40^d	1	40^d	1
	25	79^c	1	21^c	1	21^c	1

	10	92.5[d]	1.5	7.5[b]	1.5	7.5[b]	1.5
A. indica (Bark) extracts	Control	100[e]	0	0a	0	0a	0
	70	64[a]	1	36[e]	1	36[e]	1
	50	74.5[b]	0.5	25.5[d]	0.5	25.5[d]	0.5
	25	86[c]	1	14[c]	1	14[c]	1
	10	95[d]	1	5b	1	5b	1

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

In aqueous extracts highest mortality was observed in leaves (56.5%) followed by seed (54%) and bark (36%) at 70mg/ml concentration. Larval mortality produced by application of extracts of 50 mg/ml, 25 mg/ml and 10 mg/ml were seed (38.5%, 21% and 9%), leaves (40%, 21% and 7.5%) and bark (25.5%, 14% and 5%) respectively and control group no mortality found among the three extracts of *A. indica* under evaluation. Aimee *et al.*, (2017) [1] determined aqueous extracts of the leaves of these plants showed toxic effects respectively on larvae and on the engorged females of *R.* (*B.*) *microplus*. Nawaz *et al.*, (2015) [8] evaluate water extract prepared from leaves of *A. indica*, *D. sisso* and *M. alba* was used to anti-tick activity of plants against 12-14 days old larvae of *R. microplus*.

Efficacy of *A. indica* (Seed, leaves and bark) in aqueous extracts against *R. (B.) microplus* in Adult immersion test (IO%): AIT was used in present study to determine the acaricidal activity against *R.*(*B.) microplus* with various concentration of *A. indica* (Seed, leaves and bark) of aqueous extract; FAO, (2004) [2]. In AIT the dependent decrease in reproductive index and increase in inhibition of oviposition was observed from concentration 10 to 70 mg/ml. A significant percentage inhibition of oviposition (IO%) at 70, 50, 25 and 10 mg/ml the extracts were seed (54.27%, 43.49%, 22.76% and 11.7%), leaves (62.05%, 33.68%, 19.57% and 5.41%) and bark (36.57, 24.32%, 14.32% and 6.7%) respectively. The reproductive index increased as the concentration of the extracts decreased from 70 mg/ml to 10 mg/ml respectively. No mortality of ticks was observed at any concentration, as shown (Table 2).

Table 2: Acaricidal efficacy of different concentrations aqueous 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	0.76	0.040	0.355	0.025	0.46[e]	0.010	0a	0.00
	70	0.58	0.05	0.122	0.016	0.21[a]	0.010	54.27[e]	3.16
	50	0.64	0.16	0.165	0.035	0.26[b]	0.010	43.49[d]	0.94
	25	0.86	0.1	0.305	0.06	0.35[c]	0.004	22.76[c]	2.76
	10	1.17	0.6	0.470	0.27	0.40[d]	0.004	11.7[b]	3.19
A. indica (Leaves) Extracts	Control	0.76	0.040	0.355[c]	0.0250	0.46[c]	0.010	0a	0.00
	70	0.7650	0.035	0.135[a]	0.0250	0.175[a]	0.025	62.05[d]	4.61
	50	0.8500	0.020	0.26[b]	0.0100	0.305[b]	0.005	33.68[c]	0.35
	25	0.8200	0.040	0.309[bc]	0.0090	0.3745[b]	0.005	19.57[b]	0.43
	10	0.8200	0.020	0.36[c]	0.0100	0.435[c]	0.005	5.41[a]	0.97
A. indica (Bark) Extracts	Control	0.76	0.04	0.355[c]	0.025	0.46[c]	0.010	0a	0.00
	70	0.59	0.03	0.173[a]	0.020	0.29[a]	0.019	36.57[e]	2.75
	50	0.61	0.03	0.214[b]	0.013	0.34[b]	0.002	24.32[d]	1.21
	25	0.685	0.01	0.27[b]	0.004	0.39[bc]	0.014	14.37[c]	1.18
	10	0.67	0.02	0.28[b]	0.01	0.42[c]	0.002	6.7[b]	1.59

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

In aqueous extracts of seeds inhibition of oviposition (54.27%, 43.49%, 22.76% and 11.7%), leaves (62.05%, 33.68%, 19.57% and 5.41%) and bark (36.57, 24.32%, 14.32% and 6.7%) respectively. AIT the decrease in reproductive index and increase in the percent inhibition of oviposition, was evident during the study. Schwalbach *et al.*, (2003) [10] explain the efficacy of a 10% aqueous Neem (*A. indica*) seed extract for tick control in small East African and Toggenburg female goat kids in Tanzania. However, Nahar (2004) reported that aqueous extract of neem showed 86.67% efficacy in vitro on spray method using 2% concentration. Acaricidal efficacy of aqueous extracts seed, leaves and bark of *Azadirachta indica* against cattle tick, *R. (B.) microplus.* The larvicidal and acaricidal effect of neem seed and leaves are probably due to azadirachtin and triterpenoids, salannin, nimbin among the different active components. In a few studies, the relationship between salivary gland degeneration and levels of ecdysteroids as well as effects of on this relationship were elucidated in cattle tick. (Mulla and Tianyun, 1999) [7].

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