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Research Article

Assessment of Antitick and Repellent Activity of *Azadirachta indica* Oil against Adults and Larvae Stages of *Rhipicephalus microplus*

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Ticks are blood-sucking parasites and occur all over the world, especially in humid and subhumid nations such as India. They are very important vectors of many economically important livestock diseases, such as *Anaplasma* spp., *Babesia* spp., and *Theileria* spp. Ticks were collected from organized and unorganized cattle farms situated in and around Mhow. The initially collected samples of ticks (≤200) were washed in tap water and identified as *Rhipicephalus microplus*. The study was carried out from June 2021 to May 2022. Ticks were treated with 0.625, 1.25, 2.50, 5, and 10% oil of *Azadirachta indica* in both the adult immersion test and larval immersion test. All the concentrations were prepared with absolute ethanol. In the present study, fipronil was used as a positive control using various concentrations (x/8, x/4, x/2, x, and 2x) and was prepared in distilled water. The LC_{50} values against fipronil were observed as 1.03 and 1.12 ppm for adults and larvae, respectively. Results of the study showed that the oil of *Azadirachta indica* was responsible for the mortality of adults (LC50 = 2.70%) and larval stages (LC50 = 1.27%) at all the concentrations used. Furthermore, it was observed that this oil was also responsible for the inhibition of oviposition and had excellent repellent activity at 10% concentration. Based on the study, we can conclude that the oil of *A. indica* can be used in an integrated tick management system to reduce the tick burden on animals.

1. Introduction

Rhipicephalus microplus, family Ixodidae, a tropical bovine tick, is an economically significant ectoparasite of animals and a major concern for milk producers in tropical and subtropical nations, including India. It causes severe economic losses due to blood loss, weight loss, direct damage to skin and hides, and serving as a vector of infectious diseases [1, 2]. In order to control this ectoparasite, various chemical groups, such as synthetic pyrethroids (SP), organophosphates (OP), formamidines, and macrocyclic lactones, are widely used. Such strategies aim to prevent damage and avert an epidemic by keeping population levels below a critical threshold. However, the widespread use of these chemicals causes serious environmental issues. Chemicals used for the control of ticks on cattle typically produce 100 gallons of residue (3-4 liters per animal), which are frequently discarded randomly, resulting in water and soil contamination [3]. Because of the chemical acaricides, toxic residues in milk and meat endanger human health [4]. Another major issue with the extensive use of chemical acaricides is the development of resistant tick populations, which results in the failure of tick control programs [5].

To combat resistance and other environmental issues associated with chemical control, efforts have been made in the recent past to develop long-term immunological methods of controlling ticks and tick-borne diseases. Researchers created and promoted two commercial vaccinations against *R. microplus* as a historic development [6]. After 10 years, a review of the vaccination's effectiveness revealed significant protection with reduced acaricide usage [7]. However, the vaccine's efficacy was found to be highly variable [8] and could not fulfill farmers' expectations. Furthermore, the vaccine does not provide significant protection against multispecies tick infestations, which is a common problem for livestock owners in Asia.

Plant extracts and essential oils are among the natural products that have been shown to have economically significant activity against cattle tick species and acaricide-resistant tick species [9–12]. Also, it has been discovered that these botanicals include a variety of active ingredients that can halt or postpone the emergence of resistance against chemical acaricides [13]. In addition, natural products provide a less expensive alternative to synthetic acaricides, and another most common benefit of using botanicals is their degradability [14]. New techniques are required to address the rise of populations of *R. microplus* resistant to commercial acaricides and the associated dangers to the environment and human health. Plant essential oils are potential options for creating novel products with acaricidal characteristics. Therefore, the present research study was conducted for the evaluation of the antitick and repellent activity of *A. indica* oil against *R. microplus*.

2. Materials and Methods

2.1. Study Design. For evaluation of the acaricidal activity of *A. indica* oil, *in-vitro* tests such as AIT, LPT, and repellency activity by choice were employed [15]. Different concentrations (2–10%) of *A. indica* oil (BiosurPharma, India) were prepared in 2% Triton X-100 (Sigma-Aldrich, USA), 10% ethanol (SDFCL, Mumbai, India), and distilled water. The stock solution (1000 ppm) of fipronil (Sigma-Aldrich, USA) was prepared in methanol (SDFCL, Mumbai, India), and working concentrations (x, 2x, 3x, and 4x) were prepared by diluting the stock solution in distilled water. The fipronil discriminative concentration is $x = 5$ parts per million (ppm). The dose-dependent bioassay was conducted to detect the lethal concentration (LC). The main purpose is to find out the minimum effective concentration.

2.2. Collection of R. microplus Ticks. Engorged female *R. microplus* was collected from different regions of the body, such as the skin of the thigh, abdomen, perineal area, and forelegs of naturally infested cross-bred cows and calves from organized and unorganized farms in Mhow, Indore (22.7196°N, 75.8577°E), Madhya Pradesh, India. The collected ticks (≤200) were pooled to form a single sample for the experiment. Ticks were examined under a stereo zoom microscope (Leica, Germany) and identified according to the taxonomic key [16]. We did not obtain any information regarding prior parasitic treatment. Engorged ticks were placed in 50 ml tubes with perforated lids to allow ventilation and then transported to the Entomology Laboratory, Department of Parasitology, College of Veterinary Science and A.H., Mhow. The collected ticks were used immediately for the adult immersion test, and some ticks from the pooled sample were maintained in an incubator at 28 ± 2°C and 80 ± 5% RH to allow egg laying and hatching.

2.3. Procurement of A. indica Oil and Acaricide. BiosurPharma supplied the *A. indica* oil from Unit 1 M.I.G-32 P.M. Nagar Rajakhedi (Maharashtra). The technical-grade fipronil was procured from Sigma-Aldrich, USA, and used for recording the resistance against it.

2.4. Evaluation of Antitick Activity of A. indica Oil against Adult R. microplus. The antitick activity of *A. indica* oil against engorged *R. microplus* ticks was determined by using the adult immersion method [17]. In brief, the thoroughly cleaned, engorged female ticks were weighed. The different concentrations of *A. indica* oil (0.625, 1.25, 2.5, 5, and 10%) were prepared in distilled water with 2% Triton X-100 and 10% ethanol. The ticks ($n = 15$) were immersed for 10 minutes in each concentration, whereas the control ticks were immersed in distilled water, and later, they were transferred to the Petri dishes padded with Whatman filter paper no. 1. Petri dishes were kept at 28 ± 2°C and 80 ± 5% RH in desiccators placed in the BOD incubator. The mortality of ticks was recorded for up to 14 days. Ticks were considered alive if they exhibited normal behavior when pressed on or physically stimulated with wooden dowels. Ticks that were incapable of movement, maintaining normal posture, leg coordination, or the absence of any signs of life were considered moribund or dead, according to Khater and Ramadan (2007). The ticks that survived after exposure to different concentrations were reared subsequently to generate data on the efficacy of *A. indica* oil on inhibition of oviposition. Researchers recorded entomological data daily for 14 days. The inhibition of oviposition was evaluated after digitally weighing the eggs using the following formula [18]:

$$\text{Reproductive Index (RI)} = \frac{\text{mean egg masses}}{\text{engorged ticks weight}}$$
$$\%\ \text{Inhibition of Oviposition (IO)} = \frac{\text{RI (control)} - \text{RI (treated)} \times 100}{\text{RI (control)}}. \quad (1)$$

2.5. Evaluation of Antitick Activity of A. indica Oil in Larvae of R. microplus. For performing the larval packet test, the method of [17] was followed with slight modifications. Approximately 100–150 larvae were placed between 3.75×8.5 cm filter papers, closed with binder clip sand, and moistened with 600 μL of *A. indica* oil at concentrations of 0.625, 1.25, 2.5, 5, and 10%. The oil was emulsified with 2% Triton X-100, 10% ethanol, and distilled water. In addition, a negative control packet was treated with distilled water, 2% Triton X-100, and 10% ethanol. Six packets were used for each treatment, and the packets were kept in desiccators and placed in a climate-controlled B.O.D. incubator at 28 ± 2°C and a relative humidity (RH) of 80 ± 5%. To prevent any possible cross-interference, the control groups were kept in different desiccators under the same conditions of temperature and humidity. After 24 hours, the packets were opened, and the number of living and dead larvae was counted. Average mortality in each packet was expressed in percentage and was calculated using the following formula: mortality (%) = (no. of dead larvae/total no. of larvae) × 100.

2.6. Repellent Activity against R. microplus. The repellent activity was assessed following the methods of [15, 19]. The *A. indica* oil was diluted in an aqueous solution containing 2% Triton X-100 and 10% ethanol. The tests were conducted on engorged female adults of *R. microplus* ticks at room temperature (28 ± 2°C). Filter papers (Whatman® qualitative filter paper, Grade 1) were cut in half (diameter 21 cm). In one half of the paper, 500 μL of the *A. indica* oil was applied using the concentrations of 5% and 10%. In the other half, the solvent solution was applied. The embedded filter papers were dried for 30 min at room temperature and then placed inside the petri dishes. Ten ticks were released at the center of each petri dish, which were then placed in the absence of light for 30 min. Petri dishes containing the solvent and distilled water were used as controls.

The repellency index (RI) was calculated according to the method of [20, 21] utilizing the following equation: $RI = 2T/(T + C)$, where T = percent of ticks attracted to the treatment and C = percent of ticks attracted to the control (solvent). The RI values varied between 0 and 2, where RI < 1 indicated a repellent effect and RI > 1 indicated an attractive effect. The RI = 1 corresponded to a neutral effect.

2.7. Statistical Analysis. For dosage response data, Graph Pad Prism-5 software was used to perform a probit analysis to determine LC_{50} and LC_{95} values [22]. The LC_{50} and LC_{95} values of *A. indica* oil were determined using regression equation analysis, and the significant values of data were analysed by one-way ANOVA.

3. Results

3.1. Susceptibility of Ticks to A. indica Oil and Fipronil. All collected *R. microplus* females from cross-breed cattle farms were tested in AIT and LPT formats. In tested ticks, 100% mortality was recorded at a 10% concentration of *A. indica* oil (Table 1). Other concentrations showed variable mortality. With the increasing concentration, reproduction index (RI) decreased and inhibition of oviposition increased at 10% concentration. The LC_{50} and LC_{95} values were recorded as 2.70 and 55.75% for AIT and 1.27 and 8.04% for LPT, respectively (Table 2).

The value of the coefficient of determination (R^2) was recorded as 0.89 and 0.87, indicating a good fit of data in the statistical model and an 89 and 87% correlated response with log doses of *A. indica* oil for AIT and LPT, respectively (Figure 1). The reproductive index showed a decreased trend with the increasing concentration of oil (Figure 2). Percent inhibition of oviposition increased with the increasing concentration of oil, as the highest inhibition was observed at 10% concentration (Figure 3).

Against fipronil, the slope value was 3.479 ± 0.4454, which is comparatively lower than the slope value of 3.67 ± 0.75 in IVRI-I, the susceptible reference strain, indicating the heterogeneity in the field populations. The resistance ratio (RR) was observed at 0.61, indicating the susceptibility of adult females. The LC_{50} value was also observed to be lower (1.03) than the reference susceptible IVRI-II strain (1.68) as all the ticks died at the maximum concentration of 4.73 ppm. The coefficient of determination (R^2) value was calculated as 0.97, indicating a good fit of data in the statistical model and a 97% correlated response with log doses of fipronil (Figure 1).

In the case of larvae, the slope was 2.719 ± 0.7459, which is comparatively lower than the slope value of 7.67 ± 2.41in IVRI-I, the susceptible reference strain, indicating the heterogeneity in the field populations. The resistance ratio (RR) was observed at 0.46, indicating the susceptibility of adult females. The lower LC_{50} value (1.12) was also observed than the reference susceptible IVRI-I strain (2.4), as all the ticks died at the maximum concentration of 4.8 ppm. The coefficient of determination (R^2) value was recorded as 0.87, indicating a good fit of data in the statistical model and an 87% correlated response with log doses of fipronil (Figure 1).

3.2. Repellent Activity against R. microplus. The repellent activity was noticed in *A. indica* oil when tested in 5 and 10% concentrations based on the description given in Table 3. The repellency indices of *A. indica* tested in the highest concentration with 30 minutes drying times are presented in Table 3, and the repellent effect of *A. indica* oil has been observed with a repellency index of 0.20 ± 0.036 which was nonsignificant.

4. Discussion

Tick control has faced significant challenges in recent decades, including the prompt development of resistance against chemical acaricides and their adverse effects on human health and the environment. According to current reports from different parts of India, the cattle tick, *R. microplus*, has developed resistance to multi-acaricides such as diazinon, deltamethrin, cypermethrin, fenvalerate, ivermectin, and amitrazand, which has been reported from various states of India such as Uttar Pradesh, West Bengal, Bihar, Gujarat,

Table 1: Dose-response data of therapeutic grade *A. indica* oil and fipronil against adults of *R. microplus*.

	Concentrations (%)	Number of ticks treated	Weight of ticks (mg) (mean ± SE)	Mortality of ticks (%)	Egg mass (mg) (mean ± SE)	RI	IO (%)
A. indica oil	0.625	15	2863 ± 0.007	13.33	626 ± 0.001	21.86	61.3
	1.25	15	2894 ± 0.003	33.33	580 ± 0.003	20.04	66.4
	2.5	15	2770 ± 0.003	60.0	523 ± 0.002	18.88	68.3
	5	15	1761 ± 0.008	73.33	280 ± 0.003	15.90	73.3
	10	15	1998 ± 0.067	86.66	154 ± 0.002	7.70	87.1
	Control	15	2929 ± 0.029	0.0	1750 ± 2.88	59.74	0.0
Fipronil	0.59	15	2477 ± 0.017	26.66	870 ± 0.010	35.12	42.4
	1.18	15	2477 ± 0.009	46.66	830 ± 0.006	33.50	45.1
	2.36	15	2437 ± 0.019	86.66	235 ± 0.008	9.64	84.20
	4.73	15	2352 ± 0.054	99.33	0.0	0.0	100
	Control	15	2767 ± 0.017	0.0	1689 ± 0.032	61.04	0.0

RI = reproductive index; IO (%) = inhibition of oviposition.

Table 2: Mortality slope, R^2, LC_{50}, LC_{95} values with 95% CI of *R. microplus* against *Azadirachta indica* oil and technical-grade fipronil by adult immersion test (AIT) and larval packet test (LPT).

Acaricides	Bioassay	Slope ± SE	R^2	LC_{50} (95% CI)	LC_{95} (95% CI)
A. Indica oil	AIT	1.248 ± 0.2523	0.89	2.70 (2.23–3.26)	55.75 (37.21–83.52)
		RI = −10.78 ± 2.619	0.84		
		% IO = 14.71 ± 0.940	0.98		
Fipronil		3.479 ± 0.4454	0.97	1.03 (0.96–1.10)	3.10 (2.70–3.55)
A. Indica oil	LPT	2.049 ± 0.2863	0.95	1.27 (1.13–1.42)	8.04 (6.32–10.22)
Fipronil		2.719 ± 0.7459	0.87	1.12 (1.03–1.22)	4.51 (3.77–5.39)

Rajasthan, Haryana, Madhya Pradesh, Punjab, Assam, and a low level of resistance from Kerala [23–32]. Along with the above-mentioned compounds, resistance to ivermectin [31, 33, 34] has also been reported in Punjab, Uttar Pradesh, and Madhya Pradesh. *R. microplus* has developed multi-acaricide resistance, and tick management is the major challenge for productive animal herd maintenance. Plant products are a rich source of bioactive organic chemicals and are advantageous over synthetic acaricides because they are less toxic, less susceptible to resistance development, and easily biodegradable. Plants offer a variety of natural compounds that can disrupt all biological processes of insects, interfering with their life cycle, and are considered a crucial component of ethno-veterinary practices [35, 36].

The current study demonstrated that *A. indica* oil had a strong acaricidal effect on adult *R. microplus,* which started on the first day of treatment and lasted up to the 14th day of treatment, causing more than 85% mortality. The LC_{50} values for adults and larvae were 2.7 and 1.27%, respectively. As compared to adults, mortality in larvae was higher at lower concentrations. Similar findings were also observed by various researchers, who recorded varying degrees of efficacy of *A. indica* extract against *R. microplus* [37–44]. A high mortality rate was also recorded in *R. microplus* by [45] after treatment with 70 mg/ml of neem seed extracts. Similarly, other species of ticks [46] recorded substantial mortality rates in *Amblyomma variegatum* at 100, 80, and 20% concentrations of neem oil extract. Other workers also reported different levels of larval mortality in different tick species (*R. pulchellus* and *R. sanguinus*) [44, 47–50].

Additionally, it was observed that *A. indica* oil affected *R. microplus* oviposition. The ability of female ticks to deposit eggs was lowered with the increasing concentration of oil. Furthermore, the reproductive index also declined gradually as the neem oil inhibited tick oviposition as well as reproductive efficiency. A similar kind of effect on oviposition was also recorded by many workers [41, 42, 51, 52]. The authors of [43, 53] also recorded reduced hatchability of *R. microplus* eggs after treatment with neem extract.

The anti-insect activity of neem oil has also been recorded against *Drosophila melanogaster*, *Gyropsylla spegazziniana*, *Chrysodeixis chalcites*, and *Aspergillus carbonarius* [54–57]. In addition, neem is also known to possess various properties such as anti-inflammatory, antiarthritic, antipyretic, hypoglycemic, antigastric ulcer, spermicidal, antifungal, antibacterial, diuretic, antimalarial, antitumor, and immune modulatory [58, 59]. Most synthetic compounds that have repellent properties have issues with their effectiveness, safety, and environmental implications [60]. In the current investigation, *A. indica* oil's repellent properties were also noted, and a 10% concentration of the oil successfully repelled adult female ticks. The mechanism of action of the repellent effect of herbal oils and essential oils is undetermined [61]. They might be working by creating a vapor barrier that prevents arthropods from coming into contact with or landing on the skin [62]. Comparable numbers of authors documented the repulsive properties of other plant oils, including *Tanacetum vulgare*, *Rosmarinus officinalis*, *Ocimum basilicum*, *Mentha piperita*, and *Citralviminalis* [63–65].

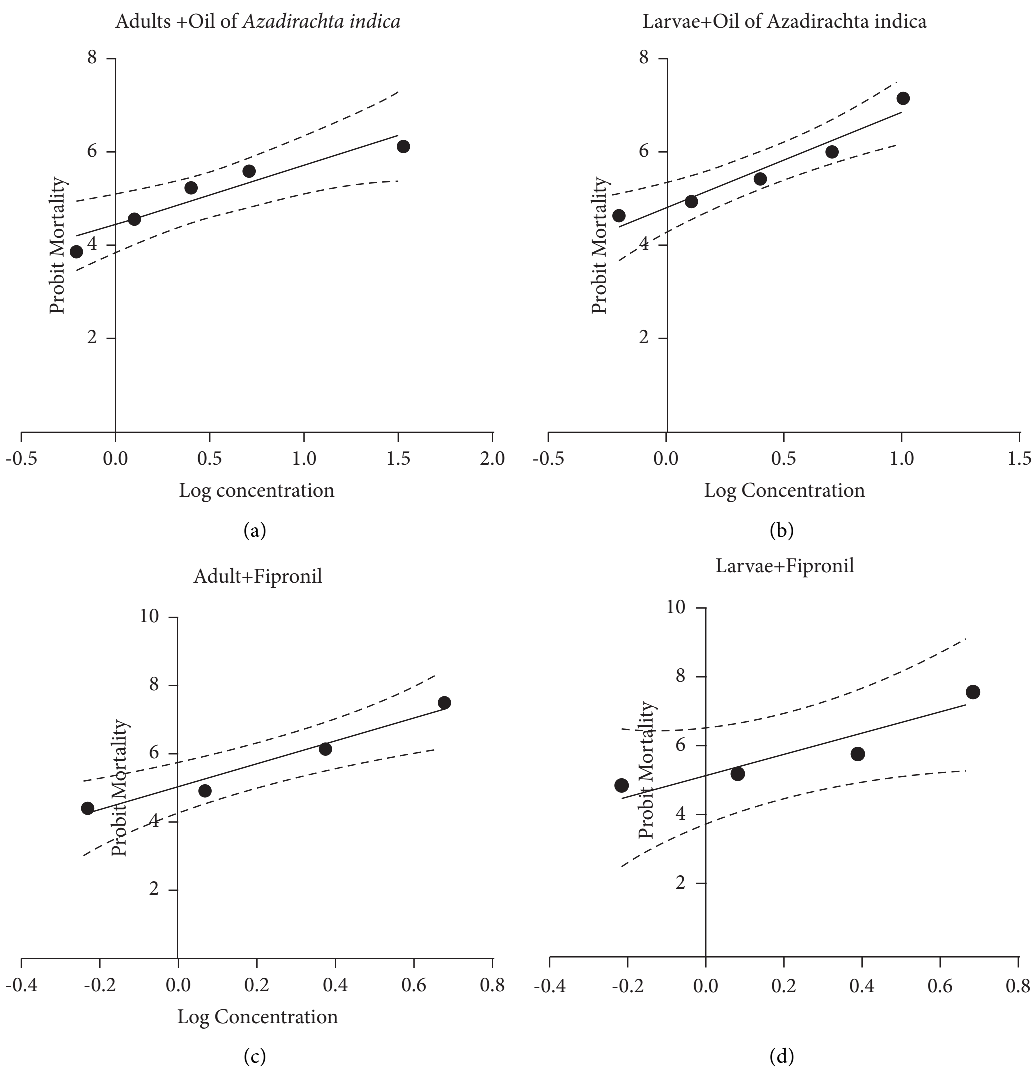

Figure 1: Regression showing probit mortality in adult immersion test (AIT) and larval immersion test (LPT) against log concentration of *Azadirachta indica* oil and fipronil in *Rhipicephalus microplus* population.

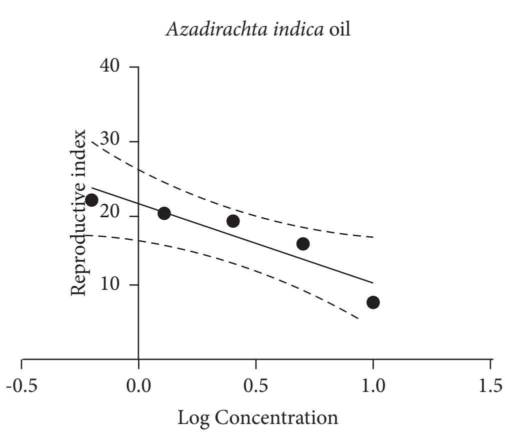

Figure 2: Regression graph between reproductive index (RI) and log concentration of (A). *indica* oil against adult of *R. microplus* ticks.

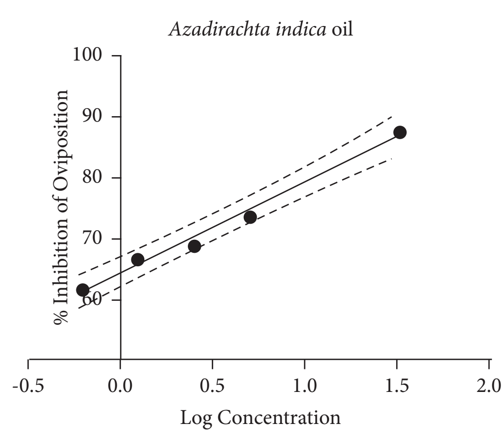

Figure 3: Regression graph between % inhibition of oviposition (IO %) and log concentration of (A). *indica* oil against adult of *R. microplus* ticks.

Table 3: Repellency indices (RI) ± standard error (SE) of the *Azadirachta indica* oil, after drying for 30 min and tested on adult females of *R. microplus*.

Particular	Concentration (%)	No. of ticks	% of ticks attracted to the treatment	% of ticks attracted to the control	Repellence index (RI ± SE)*	Classification**
Azadirachta indica oil	5	10	45	55	1.10 ± 0.034	Neutral
	10	10	10	90	0.20 ± 0.036	Repellent

RI < 1.0 indicated a repellent effect and RI > 1.0 indicated an attractive effect. RI = 1.0 corresponded to a neutral effect. *RI ± SE = repellency index ± standard error. **Based on 1.0 ± standard error for each treatment according to Girao Filho et al. (2014) and Xavier et al. (2015).

Fipronil is the least-used acaricide in the Indian cattle industry. All of the tested *R. microplus* ticks were susceptible to this drug, and the results were consistent with those recently reported by [66] utilizing AIT and LPT. Reported cases of fipronil resistance in Brazil, Uruguay, and Mexico [67–69] are rare overall due to the compound's limited use in many countries. Regular resistance monitoring is essential to limit the spread of resistance populations [70].

5. Conclusion

The oil of *A. indica* was found to be a powerful acaricidal drug and has powerful repellent action on adult *R. microplus* females. Therefore, it appears to be a promising drug for future formulations intended to reduce tick infestation. In order to advance in the quest for effective acaricides and repellents, additional research should be conducted to investigate the toxicity of this oil on mammals and nontarget organisms. As the current study demonstrated the effectiveness of fipronil against larval and adult stages of ticks, its judicious use is mandatory for effective tick management at the field level.

Data Availability

Raw data were generated at the Entomology Laboratory, Department of Parasitology, College of Veterinary Science and Animal Husbandry, Mhow (NDVSU), Indore, Madhya Pradesh. Derived data supporting the findings of this study are available from the corresponding author on request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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