

RESEARCH PAPER

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Comparative *in vitro* evaluation of the biocidal potential of neem (*Azadirachta indica*) and tobacco (*Nicotiana tabacum*) ethanolic leaf extracts against dog tick (*Rhipicephalus sanguineus*) and cat flea (*Ctenocephalides felis*)

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**Key words:** Neem leaf, Tobacco leaf, *Ctenocephalides felis*, *Rhipicephalus sanguineus*

**Received:** May 20, 2026    **Accepted:** June 04, 2026    **Published:** June 08, 2026

**DOI:** <https://dx.doi.org/10.12692/ijb/28.6.51-59>

ABSTRACT

This study was incubated to investigate the species specific ectoparasitic activity of Neem (*Azadirachta indica*) and Tobacco (*Nicotiana tabacum*) ethanolic leaf extracts against canine tick (*Rhipicephalus sanguineus*) and feline flea (*Ctenocephalides felis*). The observed effects were attributed to the presence of bioactive secondary metabolites, quantitatively identified as coumarins, flavonoids, phenols, saponins, and tannins, known to interrupt nervous signaling, membrane protection, and digestion processes in arthropods. This research determined a clear, specific response of parasites to the tested extract preparations, highlighting distinct acaricidal and fleacidal dynamics. In dog ticks, mortality was suggestively influenced equally by extract formulation and exposure period, signifying a time-dependent and treatment-dependent acaricidal action, showing predominantly swift and sustained lethal effect comparable to the commercial formulation, underscoring its strong potential for tick control applications. Cat fleas, in contrast, showed unvaryingly high death rate across all treatment groups and contact periods, with insignificant effects observed ( $p > 0.3$ ), signifying that the anti-flea response had reached a maximal or ceiling response characterized by instantaneous and broad-spectrum efficacy. Collectively, these results underscore that ectoparasite vulnerability to tobacco and neem extracts differ by species *in vitro*, with ticks selectively responding with formulation type and time of exposure, whereas fleas are extremely vulnerable across all tested extracts, comparable to commercial formulations.

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## INTRODUCTION

Infestations caused by ticks and fleas in Southeast Asia differ by country and host species. These parasites spread zoonotic pathogens, including *Rickettsia*, *Bartonella*, *Babesia*, *Ehrlichia*, and *Hepatozoon*, which all pose significant public health risks. Notwithstanding the absence of economic statistics, infestations and their related pathogenic agents may indirectly injure the economy and community health. Data from nations display a lot of variance. The account articulates that 89.1% of cats in Malaysia had fleas, while 80% of dogs and 54.8% of cats in the Philippines were similarly infested (Colella *et al.*, 2020). Molecular analysis of 392 ticks and 248 fleas from Southeast Asian companion dogs and cats showed that 9.4% of ticks bear at least one pathogen, mostly *Hepatozoon canis* (5.4%), followed by *Ehrlichia canis*, *Babesia vogeli*, *Anaplasma platys*, and others. Of the fleas surveyed, 42.7% had pathogens (Nguyen *et al.*, 2020). Blood and flea specimens from 21.5% of flea infested cats showed *Bartonella* spp. DNA, with *Bartonella henselae* being more common than *B. clarridgeiae*. Fleas from cats showed a significantly higher probability of concealing *Bartonella* spp. in comparison to those from dogs (Zarea *et al.*, 2022). In urban Vientiane (Laos), *Rickettsia asembonensis* and *R. felis* were surveyed in 44.4% of pooled flea samples, while *Rickettsia* spp. were found in 3.5% of tick congregates. *Ehrlichia canis* and *Anaplasma platys* were seen in 22.5% of ticks taken from dogs (Phommadeechack *et al.*, 2025). Cases of human illnesses are also documented, wherein contagions with *Rickettsia felis* have been documented in Thailand, Laos, and other parts of Asia, but they are probably underdiagnosed as the indications are nonspecific and the unavailability of adequate analytical apparatuses (Edouard *et al.*, 2014). An investigation in Malaysia has found that 50.5% of feline owners had a modest level of familiarity with fleas, and 65.3% had a low level of understanding as to how these diseases can spread to susceptible hosts (Azrizal-Wahid *et al.*, 2022).

The primary technique of controlling cat fleas and dog ticks is through the use of topically applied products, orally taken medications, or application of

environmental preparations of these insecticides and acaricides. Several commercial products can be employed to eliminate these pests, ranging from organic phosphates, pyrethroids, phenylpyrazoles (like fipronil), macrocyclic lactones, and isoxazolines (Makwarela *et al.*, 2025). Still, cat fleas demonstrated resistance to a number of various kinds of chemicals, including organophosphates, carbamates, pyrethroids or pyrethrins, organochlorines, and even fipronil. Accordingly, Florida field strains showed resistance ratios of about 6.8 towards cyfluthrin, 5.2 to cypermethrin, and 4.8 for fluvalinate in contrast to California strains (Coles and Dryden, 2014). Lately, fleas with L1014F and T929V mutations in the para gene were not susceptible to flumethrin, but they were affected by fipronil, imidacloprid, and propoxur (Rust *et al.*, 2015).

A report in the UK indicates that fipronil and imidacloprid, two common flea medications for pets, remain in the environment, and such compounds were demonstrated in most songbird nests, which are directly associated with unhatched eggs and chicks death (Carrington, 2025). An investigation of about 3,861 aquatic trials from 20 English rivers between 2016 and 2018 displayed that fipronil and its metabolites were exemplified in 98.6% of the samples, with usual concentrations 5.3 to 38 times higher compared to chronic toxicity limits (Perkins *et al.*, 2021). Adulteration was also found in pet-friendly ponds, wherein the presence of fipronil and imidacloprid caused a damaging impact to invertebrates, which were wholly examined in dog-access regions (Yoder *et al.*, 2024).

Notably, a research finding of 98 treated dogs found that bathing, bedding washes, and handwashing liberate up to 24.5% of fipronil and 16.8% of imidacloprid into wastewater (Perkins *et al.*, 2024). An examination of swimming emissions denoted that dogs that were given spot-on treatments can release up to 0.4% to 4% of fipronil and 1.4% to 10% of imidacloprid into water for a period of 28 days post-treatment, which also poses a hazard to the environment (Perkins *et al.*, 2025). Even though inclusive toxicological statistics in humans are

incomplete, these findings infer potential health menace, together with relationships between fipronil contact and heightened possibilities of diabetes, high blood pressure, and evolving complications in newborns (Goulson, 2024). Also, an analysis articulates that fipronil metabolites, like fipronil-sulfone, can attach to mammalian GABA receptors and bind in tissues, and imidacloprid products, corresponding desnitro imidacloprid, which are more neurotoxic to non-target species (De Marchi *et al.*, 2025).

Regulating organizations often discount the threats to the environment that come with the application of animal ectoparasiticides, unlike agricultural compounds (De Marchi *et al.*, 2025). Supervisory agendas are covering behind discovered evidence, and there is swelling stimulus to reassess and lessen the environmental hazards linked with veterinary parasiticides. According to Oliveira *et al.* (2022), the uncontrolled use of synthetic insecticides and acaricides causes pests to become resistant and injury to the environment. This tells how vital it is to search for safer alternatives, naturally occurring selections for animals, humans, and the environment. Pet owners and veterinary doctors are progressively searching for natural and safer means to eliminate fleas and ticks due to the safety impact, mounting of resistance, and environmental detrimental effects of commercially available inorganic chemicals (Banuls *et al.*, 2023).

Compounds in botanical plants possess numerous dynamic elements, which render pests less unaffected to substances with specific targets (Jesus *et al.*, 2025). Indeed, there are still problems to unravel beforehand on laboratory triumph of plant extracts can be turned into practical, available, and harmless formulations for utilization in the real world, but high potential as an alternative. Preparations when aptly formulated and judiciously applied, these botanical formulations will display considerable efficacy against cat fleas and dog ticks, while instigating nominal adverse side reaction on animals with negligible environmental impact, thus providing a viable alternative to synthetic veterinary chemicals.

## MATERIALS AND METHODS

### Plant preparation

Tobacco (*Nicotiana tabacum*) and Neem (*Azadirachta indica*) leaves were used in the experimental assays, considering their widespread utilization and historical therapeutic implications. The Bureau of Plant Industry (BPI) in Manila, Philippines, verified each plant species' specimens to ascertain the herbal identity. The Institutional Animal Care and Use Committee (IACUC) at Cagayan State University ethical rules certification was sought prior to actual experimentation.

### Plant extraction

Wilkins® distilled water was utilized to rinse off the leaves and eliminate possible contaminants and debris. The plant samples were air dried under room temperature for seven days, and cut into small pieces and crushed into a fine powder with an electric blender. A flour sieve was utilized to make the powdered material all the same size and kept in airtight, labeled containers at 4 °C until ready for extraction. For the ethanolic extraction procedure, 100 g of each powdered plant was placed into an Erlenmeyer flask containing 1,000 mL of 95% ethanol. The mixtures were soaked at room temperature (about 27 °C) for 16 hours covered with aluminum foil and then regularly stirred every 2 hours to obtain the bioactive plant components. The filtrates were concentrated under reduced pressure using a Genevac EZ-2 series rotary evaporator to remove the solvent after three passes through Whatman No. 1 filter paper. The crude extracts were kept frozen until ready for use.

### Qualitative phytochemical analysis

Qualitative phytochemical screening of the ethanolic extracts was conducted at the Analytical Laboratory of Cagayan State University, Philippines.

### Collection of flea and tick specimen

Under controlled conditions, animals were appropriately restrained. Using a powder-free sterile gloves, adult *Rhipicephalus sanguineus* and *Ctenocephalides felis* were manually collected from

naturally infested dogs and cats, correspondingly. To guarantee uniformity and condense operator bias, tick specimens were gathered with a sterilized tweezer. On the other hand, with the use of frozen ice gel pack, this was smoothly rubbed against the cat's coat to let fleas freeze and fall following a standardized sampling protocol. Fleas were collected using standardized entomological procedures designed to minimize handling stress and collection variability (Beugnet *et al.*, 2014). All recovered parasites were immediately placed in their labeled chambers ready for experimental manipulation.

### Treatment application

One milliliter of the prepared plant extract solution was evenly infused over a 30 mm Petri dish for each test. Prior putting in the tea bag filled with the desired number of dog ticks and cat fleas, the treated filter papers were left to air dry for 5-10 minutes in a sterile environment to mimic exposure. This pre-drying step makes sure that the doses and exposures are the same across all samples.

### Test chamber setup

In the controlled bioassay, 10  $\mu\text{m}$  microporous tea bags were utilized to hold dog ticks and cat fleas while letting the treatment solution spread. This serves as a membrane that enables controlled-release and containment assay systems, surround organisms and let active agents move through them. Then, each tea bag was assigned in one of six test solutions: ethanolic leaf extracts of tobacco and neem, citronella essential oil, a mixture of all three botanical treatments, and two commercial preparations of anti-tick and anti-flea shampoos.

### Experimental design

The study made use of two distinct ectoparasites, namely, dog tick (*Rhipicephalus sanguineus*) and cat flea (*Ctenocephalides felis*) to individualistically evaluate the efficacy of the test solutions. A total of 180 ectoparasites from each species were employed, and each species was handled discretely during the experiments to evade cross-contamination and make it easier to precisely qualify species-specific

responses. In a completely random design, each parasite species was assigned into one of six treatment groups, with 30 parasites in each group. This design made it possible to look at the effects of treatment on ticks and fleas distinctly, while also keeping the statistics correct employing enough replications.

Tea bags that had been soaked in the respective test solutions were used to make arenas for both parasite species. There were 18 arenas in total for each tested species, encompassing six treatment groups (T1–T6) repeated three times (R1–R3). All arenas were kept in a controlled environment under room temperature and relative humidity.

### Exposure and monitoring

The bioassays were done at a temperature of  $27 \pm 2$  °C and a relative humidity of  $70 \pm 5\%$ . Ticks and fleas mortality were checked by poking the parasites every three hours for 24 hours using a stereomicroscope. Mortality was recorded if there were no movements observed.

### Data collection and analysis

ANOVA was used to determine whether significant differences existed among the treatment means at 5% level, and employed a Generalized Linear Model (GLM) to look at both the main effects and the interaction effects of extracts on killing ticks and fleas over time.

## RESULTS AND DISCUSSION

### Qualitative phytochemical screening

This study examined qualitatively the presence of specific phytochemicals in ethanolic leaf extracts of *Azadirachta indica* (Neem) and *Nicotiana tabacum* (Tobacco). The ethanolic extract of *A. indica* was found positive for the following secondary metabolites, namely: coumarins, flavonoids, phenols, saponins, and tannins, while *N. tabacum* only has coumarins, flavonoids, and tannins. Earlier studies have corroborated the antiparasitic potential of botanical-derived phytochemicals. The anthelmintic activity of *Matricaria recutita* extract against *Haemonchus*

*contortus* was linked to the positive demonstration of polyphenol, flavonoids, and tannins, which are known to affect the lifespan of the parasites (Hajaji *et al.*, 2018). Likewise, *Camellia oleifera* and *Atriplex laciniata* saponins display undeniable insecticidal and anthelmintic efficacy against diverse pests by damaging the shield of tissues and leading to high death even at modest dosages (Cui *et al.*, 2019; Zul *et al.*, 2017). A systematic investigation of 44 studies further revealed that flavonols have broad-spectrum antiparasitic effects against several protozoan parasites, substantiating their potential as natural antiparasitic agents (Mohd Sabri *et al.*, 2025; Clavin *et al.*, 2016). Likewise, coumarins from *Peucedanum* species have been shown to limit the development and nourishment of *Spodoptera littoralis* larvae, signifying the implication of coumarin derivatives as prospective botanical pesticides (Hadaček *et al.*, 1994; Tang *et al.*, 2024).

#### ***In vitro* acaricidal efficacy**

Repeated-measures ANOVA (Table 1) suggests a highly significant effect of exposure duration on tick mortality ( $F = 97.18$ ,  $p < 0.001$ ), signifying a liberal intensification in mortality over the 24-hour observation period, irrespective of treatment. A significant time and treatment interaction was detected ( $F = 9.48$ ,  $p < 0.001$ ), indicating that the mortality pattern and rate over time exhibited considerable variation among treatment groups. This interaction shows that some treatments had faster or stronger acaricidal effects at certain exposure times. Furthermore, the analysis of between-subjects effects demonstrated a significant main effect of treatment ( $F = 28.40$ ,  $p < 0.001$ ), indicating that tick mortality exhibited substantial variation across the assessed treatments. The findings collectively indicate that both exposure duration and treatment type are critical in determining acaricidal efficacy against dog tick (*Rhipicephalus sanguineus*), with treatment-specific variations becoming increasingly evident with prolonged exposure time.

The logistic regression model (Fig. 1) that included treatment and exposure time explains about 83% of

the differences in tick mortality that were seen ( $R^2 \approx 0.83$ ,  $p < 0.001$ ). Mortality went up a lot with longer exposure times, showing a clear dose-dependent temporal effect ( $p < 0.001$ ). When Treatment 1 (Neem leaf ethanolic extract) was used as a reference, Treatments 3 (Commercial Citronella essential oil) and Treatment 5 (Commercial Acaricide A) caused much higher death as exposure time was extended. This means that they were more effective at killing ticks as contact period is prolonged. There were no significant statistical differences between Treatments 2 (Tobacco leaf ethanolic extract) and Treatment 4 (Mixture of Neem leaf ethanolic extract and Tobacco leaf ethanolic extract) and Treatment 6 (Commercial Acaricide B), which imply they had analogous biological effects.

Statistically, repeated-measures ANOVA and logistic regression showed significant effects of treatment and exposure time, as well as significant time and treatment interactions, demonstrating that mortality was time-dependent and that certain treatments, principally Treatments 3 and 5, which elicited considerably higher tick mortality. This indicates that these treatments are effective acaricidal agents, capable of inducing rapid and sustained tick kill over the 24-hour period.

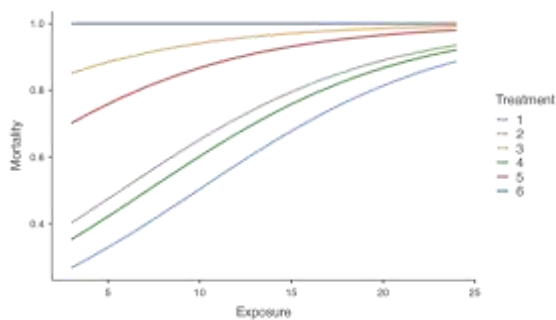
Repeated-measures ANOVA (Table 2) showed that the tested solutions (T1–T6) reliably exerted their biological effects against cat flea (*Ctenocephalides felis*) during the 24-hour observation period. Even though the period of contact did not yield a statistically substantial intensification in death rate ( $F = 0.87$ ,  $p = 0.534$ ), this infers that the treatments were able to cause their effects early in the exposure time and preserve this action over time. Equally, the absence of a considerable interaction among treatment and exposure time ( $F = 1.03$ ,  $p = 0.437$ ) specifies that all treatment groups revealed an analogous and stable pattern of effectiveness, reflecting the constant biological action of the tested formulations across the observation period.

**Table 1.** Effects of neem and tobacco ethanolic leaf extracts and exposure time on *in vitro* mortality of *Rhipicephalus sanguineus* (repeated-measures ANOVA)

Source of variation	df	F-value	p-value
Exposure time (h)	7	97.18	<0.001
Treatment	6	28.40	<0.001
Exposure time × Treatment	42	9.48	<0.001

**Table 2.** Effects of neem and tobacco extract treatments and exposure time on *in vitro* mortality of *Ctenocephalides felis* (repeated-measures ANOVA)

Source of variation	df	F-value	p-value
Exposure time (h)	7	0.87	0.534
Treatment	6	1.09	0.416
Exposure time × Treatment	42	1.03	0.437

**Fig. 1.** Logistic regression showing interaction between time exposure and solution (treatments) on the proportion killed against *Rhipicephalus sanguineus*

Treatment 1: Neem leaf ethanolic extract (NLEE)

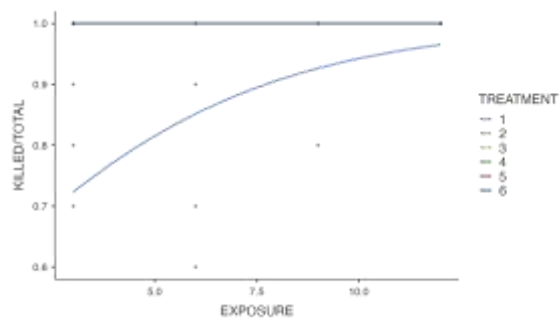
Treatment 2: Tobacco leaf ethanolic extract (TLEE)

Treatment 3: Commercial Citronella essential oil (CEO)

Treatment 4: Mixture of Neem leaf ethanolic extract and Tobacco leaf ethanolic extract (Combo solution)

Treatment 5: Commercial Acaricide A

Treatment 6: Commercial Acaricide B

**Fig. 2.** Logistic regression showing interaction between time exposure and solution (treatments) on the proportion killed against *Ctenocephalides felis*

Treatment 1: Neem leaf ethanolic extract (NLEE)

Treatment 2: Tobacco leaf ethanolic extract (TLEE)

Treatment 3: Commercial Citronella essential oil (CEO)

Treatment 4: Mixture of Neem leaf ethanolic extract and Tobacco leaf ethanolic extract (Combo solution)

Treatment 5: Commercial Fleacide A

Treatment 6: Commercial Fleacide B

Fig. 2 substantiates the robust biological effectiveness of the tested solutions (T1–T6) against cat flea (*Ctenocephalides felis*), as proved by the high explanatory power ( $R^2 \approx 0.824$ ), signifying that treatments principally accounted for the observed death. The near-ceiling forecasted mortality ( $\sim 100\%$ ) echoes a compelling insecticidal result, signifying fast and effectual lethal action upon contact.

The absence of substantial effects of exposure time ( $\chi^2(1) = 0.88$ ,  $p = 0.347$ ) and treatment type ( $\chi^2(5) = 6.03$ ,  $p = 0.304$ ) specifies that all formulations constantly produced high mortality irrespective of duration or group. These findings prove fast-acting and uniformly effective biological activity across all tested treatments.

## CONCLUSION

Taken together, these findings highlight that the effectiveness of treatments is both species and context-dependent. While some treatments display clear discrepancies in their effects on dog ticks, the same treatments yielded uniformly high lethality against cat fleas, supporting their potential as effective ectoparasitocidal agents for rapid tick and flea control. This underscores the importance of species-specific evaluation when selecting biocidal interventions and supports the use of highly effective formulations for rapid control in tick and flea infested environments.

## ACKNOWLEDGEMENTS

The author extends their gratitude to the RDE office of Cagayan State University for the financial support and

the opportunity to conduct research on animal health, principally in the field of complementary alternative medicine.

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