



UNIVERSITI PUTRA MALAYSIA

**A COMPARATIVE STUDY OF THE ACARICIDAL EFFECTS
BETWEEN TOBACCO (*Nicotiana tabacum*) AQUEOUS EXTRACT
AND FIPRONIL ON THE BROWN DOG TICK (*Rhipicephalus
sanguineus*)**

AVRIL LIM SHU TING

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FPV 2023 108**

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FACULTY OF VETERINARY MEDICINE

UNIVERSITI PUTRA MALAYSIA

SERDANG, SELANGOR

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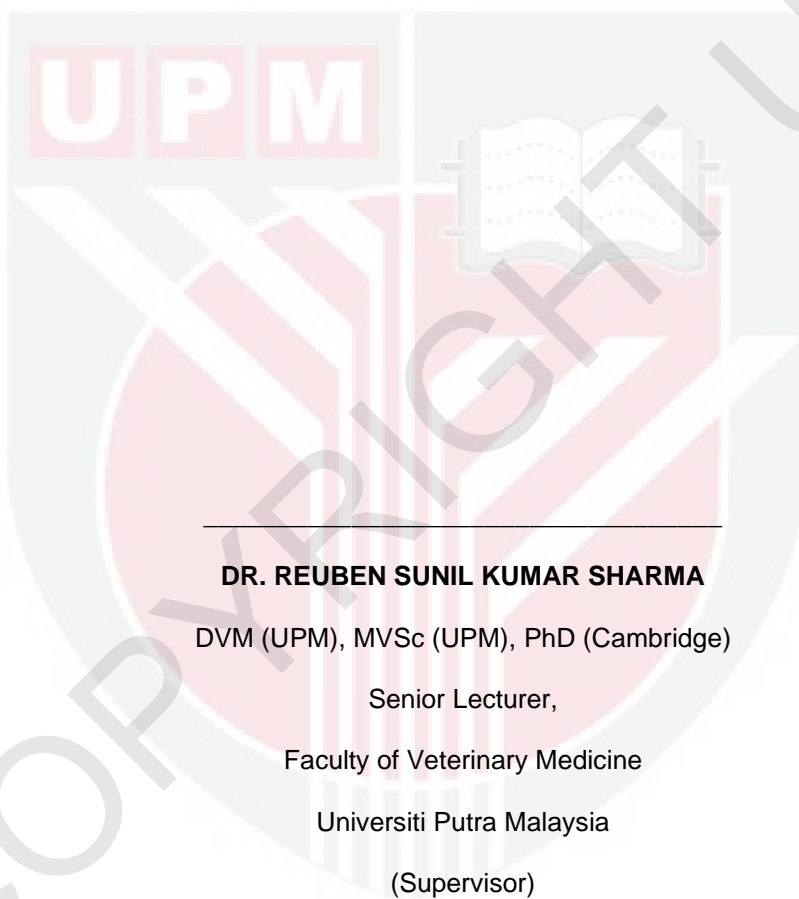
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(*Nicotiana tabacum*) AQUEOUS EXTRACT AND FIPRONIL ON THE BROWN DOG TICK
(*Rhipicephalus sanguineus*)**

AVRIL LIM SHU TING

A project submitted to the
Faculty of Veterinary Medicine, Universiti Putra Malaysia
In partial fulfillment of the requirement for the
DEGREE OF DOCTOR OF VETERINARY MEDICINE
Universiti Putra Malaysia
Serdang, Selangor Darul Ehsan.

October 2023

It is hereby certified that I have read this project paper titled “**A Comparative Study of Acaricidal Effects Between Tobacco (*Nicotiana tabacum*) Aqueous Extract and Fipronil on the Brown Dog Tick (*Rhipicephalus sanguineus*)**” by Avril Lim Shu Ting and in my opinion it is satisfactory in terms of scope, quality, and presentation as partial fulfillment of the requirement for the course VPD 4901 – Project.



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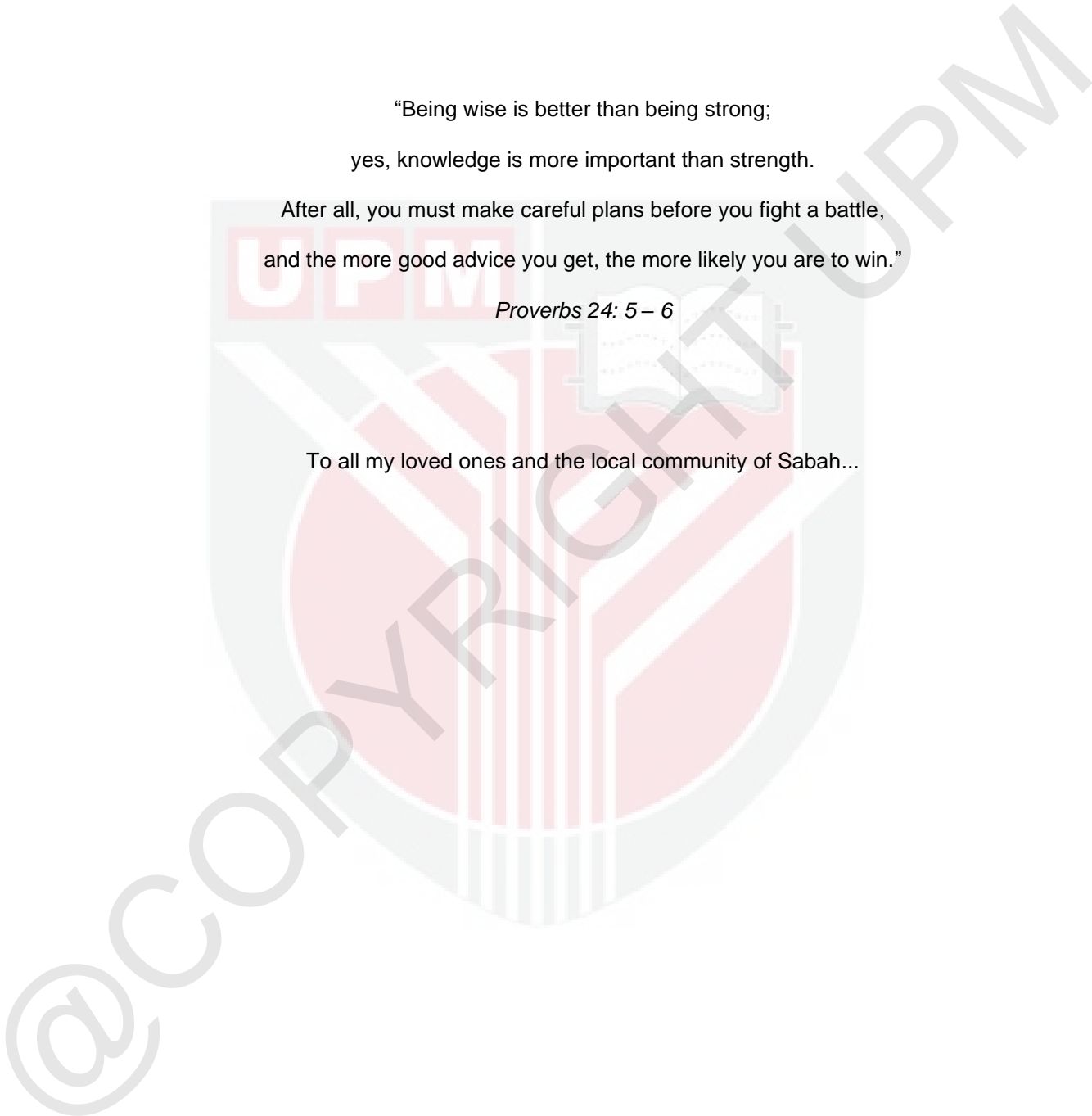
DEDICATION

“Being wise is better than being strong;
yes, knowledge is more important than strength.

After all, you must make careful plans before you fight a battle,
and the more good advice you get, the more likely you are to win.”

Proverbs 24: 5 – 6

To all my loved ones and the local community of Sabah...



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LIST OF ABBREVIATIONS

°C	Degree Celsius
%	Percentage
mm	Millimeter
mL	Milliliter
mg/mL	Milligram per milliliter
mins	Minutes
w/w	Weight in weight (Percent weight of substance by total weight)

ABSTRAK

Abstrak daripada kertas projek yang dikemukakan kepada Fakulti Perubatan Veterinar untuk memenuhi sebahagian daripada keperluan kursus VPD 4901 – Projek.

**KAJIAN PERBANDINGAN KESAN PEMBUNUHAN SENGENIT ANTARA EKSTRAK
AKUEUS TEMBAKAU (*Nicotiana tabacum*) DAN FIPRONIL PADA SENGENIT ANJING
PERANG (*Rhipicephalus sanguineus*)**

Oleh

Avril Lim Shu Ting

2024

Penyelia: Dr. Reuben Sunil Kumar Sharma

Sengkenit anjing perang, *Rhipicephalus sanguineus*, yang ditemui di seluruh dunia, kebanyakannya mengigit dan menghisap darah anjing, dan kadangkala mamalia lain termasuklah manusia. Ia terkenal dengan penularan penyakit bawaan sengkenit yang membimbangkan kesihatan awam dan alam veterinar. Kawalan sengkenit secara kimia telah menyebabkan kejadian rintangan sengkenit terhadap racun akari, keracunan anjing dan pencemaran alam sekitar. Oleh itu, alternatif yang selamat terhadap alam sekitar seperti sebatian etnobotani perlu diperkenalkan bagi kegunaan mamalia peliharaan kecil. Kajian ini dijalankan untuk menyelidiki kesan pembunuhan sengkenit secara *in vitro* dengan menggunakan larutan ekstrak akueus tembakau (*Nicotiana tabacum*) pada kepekatan yang berbeza, dan membandingkan keberkesanannya dengan racun akari komersial, fipronil. Sengkenit anjing perang dikutip dari persekitaran tempat perlindungan anjing, dan disimpan secara hidup-hidup dalam tiub *falcon*

dengan pengudaraan. Tembakau kering diperoleh dari pasar tempatan di Penampang, Sabah dan ditumbuk menjadi serbuk. Larutan stok *N. tabacum* (100%, 250mg/mL) telah disediakan dengan mencampurkan serbuk dalam air suling yang disterilisasikan. Sebanyak 70 kutu dengan bilangan yang sama bagi betina dan jantan dibahagikan sama rata kepada tujuh kumpulan rawatan yang terdiri daripada lima kepekatan ekstrak akueus tembakau (100%, 50%, 25%, 12.5% dan 6.25%), fipronil (0.29%, w/w), dan kawalan (air suling yang disterilisasikan). Rawatan telah diulangi sebanyak empat kali untuk dua rejim pendedahan (Sampul dan Rendaman). Ujian Sampul (US) dilakukan dengan mengapit sengkenit di antara dua kertas turas yang direndam dengan larutan masing-masing, manakala dalam Ujian Rendaman (UR), sengkenit dicelup ke dalam larutan masing-masing selama lima saat, kemudian diletakkan di atas kertas turas dalam piring petri dengan pengudaraan. Kematian sengkenit direkodkan pada selang masa yang berbeza dalam tempoh 24 jam. Analisis *Kruskal-Wallis* digunakan untuk membandingkan peratusan kematian, LT_{50} dan LD_{50} . Keputusan daripada kedua-dua rejim pendedahan membuktikan bahawa ekstrak akueus tembakau mempunyai kesan pembunuhan sengkenit. Selepas 24 jam, 100% sengkenit mati dalam US bagi semua kepekatan ekstrak kecuali 6.25%, dan semua ekstrak mempunyai perbezaan signifikansi ($P < 0.05$) apabila dibandingkan dengan kawalan. Kepekatan ekstrak 100% menunjukkan kadar kematian tertinggi dalam UR. Kesan pembunuhan sengkenit oleh ekstrak adalah bergantung kepada kepekatan dan keberkesannya adalah setanding dengan fipronil kerana LT_{50} bagi 100% ekstrak akueus tembakau jauh lebih pendek daripada fipronil. LD_{50} bagi ekstrak dalam US dan UR adalah masing-masing 5.48mg/mL dan 128.93mg/mL. Kesimpulannya, ekstrak akueus *N. tabacum* mempunyai potensi untuk digunakan sebagai racun pembunuh bagi kawalan *R. sanguineus*, dan keberkesannya adalah setanding dengan racun akari komersial, fipronil.

Kata Kunci: Racun akari, *Nicotiana tabacum*, ekstrak, fipronil, *Rhipicephalus sanguineus*

ABSTRACT

An abstract of the project paper presented to the Faculty of Veterinary Medicine in partial fulfillment of the course VPD 4901 – Project.

**A COMPARATIVE STUDY OF THE ACARICIDAL EFFECTS BETWEEN TOBACCO
(*Nicotiana tabacum*) AQUEOUS EXTRACT AND FIPRONIL ON THE BROWN DOG TICK
(*Rhipicephalus sanguineus*)**

By

Avril Lim Shu Ting

2024

Supervisor: Dr. Reuben Sunil Kumar Sharma

The Brown dog tick, *Rhipicephalus sanguineus*, is found worldwide, and predominantly feed on dogs and occasionally other mammals including humans. It is notable for tick-borne diseases transmission of public health and veterinary concern. Chemical tick control has led to the development of tick resistance, host intoxication and environmental pollution. Hence, environmentally safe alternatives such as ethnobotanic compounds should be introduced for small pet mammals use. This study was undertaken to investigate the *in vitro* acaricidal effects of aqueous extract of tobacco (*Nicotiana tabacum*) at different concentrations, and compare its efficacy with a commercial acaricide, fipronil. Brown dog ticks were collected from the environment of a dog shelter, and kept alive in ventilated falcon tubes. Dried *N. tabacum* was procured from a local market in Penampang, Sabah and grounded into powder. Stock solution of *N. tabacum* (100%, 250mg/mL) was prepared by diluting the powder in sterilised distilled water. A total of 70

ticks with equal numbers of females and males were equally divided into seven treatment groups comprising five concentrations of tobacco aqueous extract (100%, 50%, 25%, 12.5% and 6.25%), fipronil (0.29%, w/w), and a control (sterilised distilled water). The treatments were done in four replicates for two exposure regimes (Envelope and Immersion). The Envelope Test (ET) was performed by sandwiching the ticks between two filter papers soaked with the respective solution, whereas in the Immersion Test (IT), the ticks were dipped into the respective solution for five seconds, then placed on a filter paper in a ventilated petri dish. Tick mortality was recorded at different time intervals over a period of 24 hours. The Kruskal-Wallis analysis was used to compare the mortality percentage, LT_{50} and LD_{50} . Results from both exposure regimes proved that the tobacco aqueous extract possessed acaricidal effects. After 24 hours, 100% tick mortality was observed in the ET for all the extract concentrations except for 6.25%, with all the extracts being significantly different ($P < 0.05$) than the control. The 100% extract concentration displayed the highest mortality rate in the IT. The acaricidal effect of the extract was concentration-dependent and its efficacy was comparable to fipronil with LT_{50} of 100% tobacco aqueous extract being much shorter than fipronil. The LD_{50} of extract in the ET and IT were 5.48mg/mL and 128.93mg/mL, respectively. In conclusion, *N. tabacum* aqueous extract exhibited potential to be used as an acaricide for *R. sanguineus* control, and its efficacy was comparable to the commercial acaricide, fipronil.

Keywords: Acaricide, *Nicotiana tabacum*, extract, fipronil, *Rhipicephalus sanguineus*

1.0 INTRODUCTION

Rhipicephalus sanguineus (Latreille, 1806), commonly known as the Brown dog tick, is a common arthropod ectoparasite of domestic dogs worldwide. It is a three-host urban tick that can survive a long period of time without feeding (Dantas-Torres, 2008; Tian, 2018). It is vital to note the medical and veterinary significance as they can transmit tick-borne diseases such as Rocky Mountain spotted fever in humans, and canine ehrlichiosis, anaplasmosis, hepatozoonosis and babesiosis (Salomon *et al.*, 2022; Dantas-Torres, 2008). In addition, their blood-sucking activities may lead to skin irritation and damage in dogs (Aziz *et al.*, 2017).

To date, various methods are being employed for effective tick control measures *via* chemical products such as shampoos, dips, powders, sprays, impregnated collars and oral medication (Dantas-Torres, 2008). Due to imprudent and excessive use of commercially available chemical acaricides, increasing development of acaricide resistance is reported, besides contributing to environmental pollution and residues in food chain (Jeyathilakan *et al.*, 2019).

Alternatively, research on non-chemical acaricides is highly recommended to mitigate the possible issues introduced by the chemical acaricides. Traditional herbs in Malaysia may serve as a good alternative since they are inexpensive, widely available and environmental friendly. In this study, the tobacco leaves, *Nicotiana tabacum*, was opted for as it has been proven traditionally to be an effective pesticide, insecticide and acaricide (Agyare *et al.*, 2013).

The present study was undertaken with the following objectives:

- a. To determine the *in vitro* acaricidal effects of the tobacco aqueous extract at different concentrations on the Brown dog tick.
- b. To compare its efficacy with a commercially available acaricide (fipronil).

The hypothesis for this study is:

H₀ : The acaricidal effect of the tobacco aqueous extract will be concentration-dependent and the optimal acaricidal concentration will be comparable to fipronil.

H_A : The acaricidal effect of the tobacco aqueous extract will not be concentration-dependent and the optimal acaricidal concentration will be inferior to fipronil.

2.0 LITERATURE REVIEW

2.1 Ticks

Ticks are obligate blood feeders and distributed worldwide as ectoparasites of wild and domestic vertebrates excluding fishes. Ticks are categorised under the Phylum Arthropoda, Class Arachnida, Subclass Acari, Order Parasitiformes, and Suborder Ixodida (Nava *et al.*, 2009). They are widely recognised as important vectors of animal pathogens. When ticks feed on their host, they take in blood and cause skin damage. Subsequently, this results in inflamed, oedematous, pruritic, erythematous, scaly skin, with or without ulceration in a certain degree. Excessive excoriation can lead to secondary bacterial infections (Abubakar *et al.*, 2019). Because of the physical damages and pruritus which may perturb the animals from expressing their normal behaviour, the productivity of the animals can be compromised (Mapholi *et al.*, 2014).

All species of ticks can be divided into three families belonging to the order Ixodida: Argasidae (193 species) comprising the genus *Argas*, *Antricola*, *Carios*, *Nothoaspis*, *Ornithodoros* and *Otobius*; Ixodidae (702 species) comprising those from the genus *Amblyomma*, *Aponomma*, *Cosmiomma*, *Dermacentor*, *Haemophysalis*, *Hyalomma*, *Ixodes* and *Rhipicephalus* and the family Nuttalliellidae, which is monotypic, i.e., it has only one distinguished species, *Nuttalliella namaqua* (Brites-Neto *et al.*, 2015). These three families have different life cycles, morphological and physiological traits.

The family Argasidae are soft ticks or argasid ticks with dorsum without chitin or scutum. The lack of scutum renders the argasid ticks with no clear sexual dimorphism in the adult stage unlike ixodid ticks. A major portion of the capitulum (head) and the mouthparts are located anteriorly on the ventral surface, and hence, not conspicuous when observed dorsally. Argasid ticks have at least two nymphal stages, each feeding on host's blood. They feed rapidly (about an hour) and detach from their host promptly (Brites-Neto *et al.*, 2015; Hoskins, 1991).

The family Ixodidae are hard ticks or ixodid ticks with dorsum partially or totally covered with chitin which plays a role in sexual dimorphism and blood feeding. In adult male, the scutum covers

the dorsum and limits the blood amount which can be ingested. In adult female, the scutum is relatively smaller and becomes largely distended when engorged. All the capitulum and the mouthparts are situated anteriorly and easily observed dorsally. In contrast to the feeding behaviour of argasid ticks, ixodid ticks remain attached to their hosts for several days during feeding (Brites-Neto *et al.*, 2015; Hoskins, 1991).

2.2 Biology of *Rhipicephalus sanguineus*

2.2.1 Distribution

The Brown dog tick (*Rhipicephalus sanguineus*) is the most prevalent tick worldwide especially in subtropical and tropical regions including Malaysia (Dantas-Torres, 2010; Kazim *et al.*, 2022). It mainly parasitises the domestic dog albeit occasionally parasitises other mammals such as cats, rodents and humans including birds (Estrada-Peña *et al.*, 2013).

2.2.2 Identification

Rhipicephalus sanguineus ticks are reddish brown, small and exhibit an elongated body shape with inornate scutum. They have hexagonal *basis capituli*, short palp pedicles, convex eyes and festoons (Gonzalez-Álvarez *et al.*, 2020).

Male and female Brown dog ticks vary in their morphological features. Figure 1 shows the morphological differences between males and females. Males exhibited a pear-shaped body with widest region at the legs IV and spiracle plate, whereas females exhibited an oval body. On the dorsal aspect, unlike males with varying sizes and distribution of punctuations, females presented with more small, regularly distributed punctuations. To distinguish between males and females, the alloscutum in females is orange in colour, creating a clear distinction from the scutum. The scutum in females covers one-third of the abdomen, meanwhile in males, it covers most of the abdomen. On the ventral aspect, males are characterised by quadrangular festoons, comma-shaped and narrowly elongated spiracle plates and subtriangular adanal plates with concave

anterior on the internal margin and rounded posterior. Conversely, females are defined by well-defined festoons, broad comma-shaped spiracle plates and wide U-shaped posterior lips genital aperture (Barker and Walker, 2014; Slapeta *et al.*, 2022).

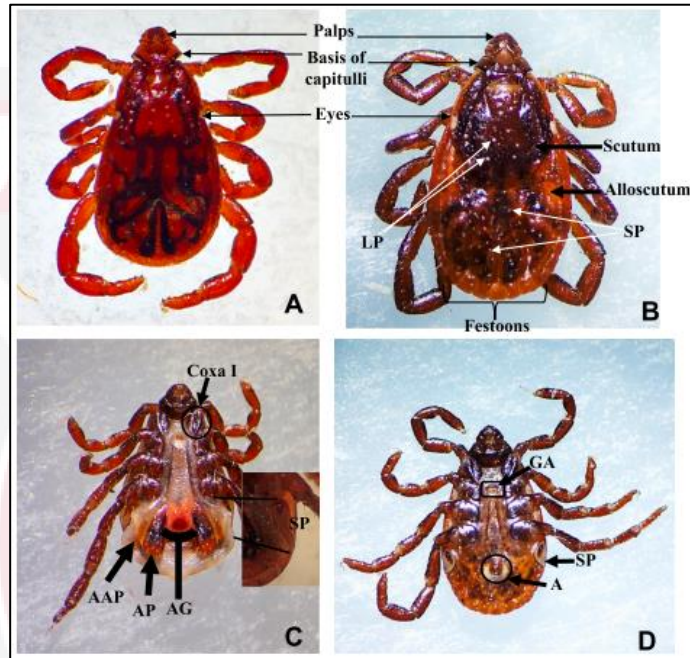


Figure 1: Morphological differences between male and female adult *R. sanguineus*.

A: Male, dorsal view. B: Female, dorsal view, LP: Large punctuations, SP: Small punctuations. C: Male, ventral view, AG: Anal groove, AP: Adanal plates, AAP: Accessory adanal plates. D: Female, ventral view, A: Anus, GA: Genital aperture, SP: Spiracular plate (Almazán *et al.*, 2023).

2.2.3 Developmental Stages

Similar to other ixodid ticks, *R. sanguineus* undergo four developmental stages, namely egg, larva, nymph and adult. The eggs are characterised by their dark brown spherical shape. The larvae are six-legged with approximately 0.54mm in length and 0.39mm in width of body size (Dantas-Torres, 2008). According to Walker *et al.* (2005), both the nymphs and adults have similar features: eight-legged, reddish-brown in colour, elongated body without ornamentation on the

dorsal. The nymphs are distinguishable from the adults by their smaller body size measuring 1.14 to 1.30mm in length and 0.57 to 0.66mm in width and are sexually immature, i.e. they exhibit no genital aperture (Dantas-Torres, 2008). The adults, which are sexually mature, are of body size of 2.28 to 3.18mm long by 1.11 to 1.68mm wide (Tian *et al.*, 2018). However, after taking blood meals, the body size of female Brown dog ticks can dramatically enlarge up to a hundred-fold (11.5mm in length and 7.5mm in width) and become gray-blue to olive (Sonenshine and Roe, 2014; Dantas-Torres, 2008).

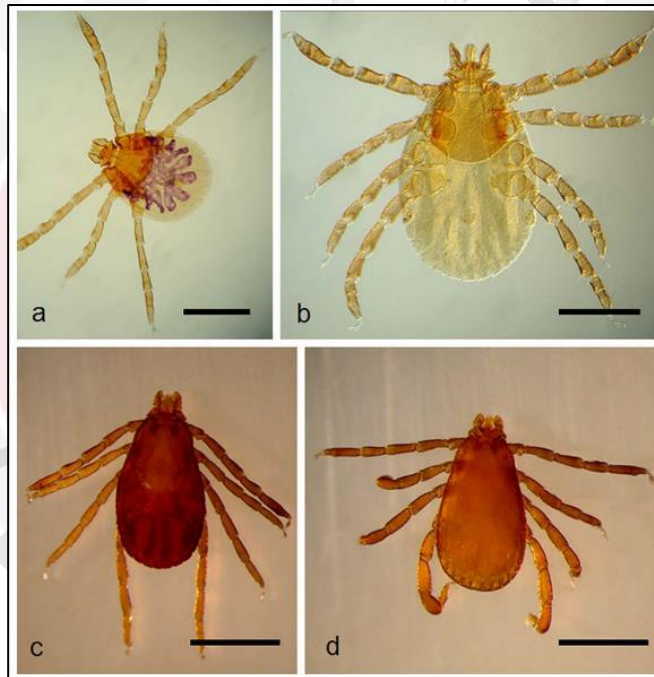


Figure 2: Immature and adult stages of *Rhipicephalus sanguineus*. a: larva (bar = 400 μ m). b: nymph (bar = 0.5mm). c: female (bar = 1mm). d: male (bar = 1mm) (Dantas-Torres, 2010).

2.3 Ecology of *Rhipicephalus sanguineus*

2.3.1 Life Cycle

Rhipicephalus sanguineus is a three-host urban tick, i.e. each active and motile developmental stage (larva, nymph and adult) undergoes blood feeding only once, then leaves

the host and undergoes ecdysis in the environment (Dantas-Torres, 2008). The life cycle can last from 6 weeks to 1 year (Foreyt, 2001).

Adult females feed on the host for 5 to 21 days and engorge. Then, they drop off the host to digest the blood meal and find a secluded area for oviposition. The ideal places are cracks and crevices in houses, buildings and dog pounds (Tian *et al.*, 2018). The average oviposition period is 16-18 days (Jittapalapong *et al.*, 2000) in an optimum temperature of 20 – 30°C (Sweetman, 1967). Engorged females can often lay over 7000 eggs with an average of 4000 eggs (Koch, 1982). The females die after oviposition (Dantas-Torres, 2008).

After egg incubation ranging from 6 to 23 days, small larvae hatch from the eggs and promptly seek for a host (Jittapalapong *et al.*, 2000; Dantas-Torres, 2008). The larvae then feed for 5 to 15 days before detaching from the host to molt into nymphs which takes about 1 to 2 weeks (Ioffe-Uspensky *et al.*, 1997). The host-seeking activity and the host attachment of the nymphs ensue and the blood feeding lasts for 3 to 13 days before dropping off the host to develop into adults (Tian *et al.*, 2018). The nymph molting duration lasts between 9 and 47 days (Pegram *et al.*, 1987).

Drop-off rhythm of Brown dog ticks is well-coordinated with host's activity. The larvae predominantly display a diurnal drop-off pattern whereby detachment from the host occurs during the daytime. In contrast, engorged nymphs and females mostly exhibit a nocturnal drop-off pattern (Jacobs *et al.*, 2004).

Brown dog ticks are notably long-lived and can survive for a long period without feeding (Tian *et al.*, 2018). Unfed larvae can survive for about 8 months, unfed nymphs 6 months and adults 19 months (Dantas-Torres, 2008). Louly *et al.* (2007) stated that the life cycle can be completed in 63 to 91 days under favourable conditions.

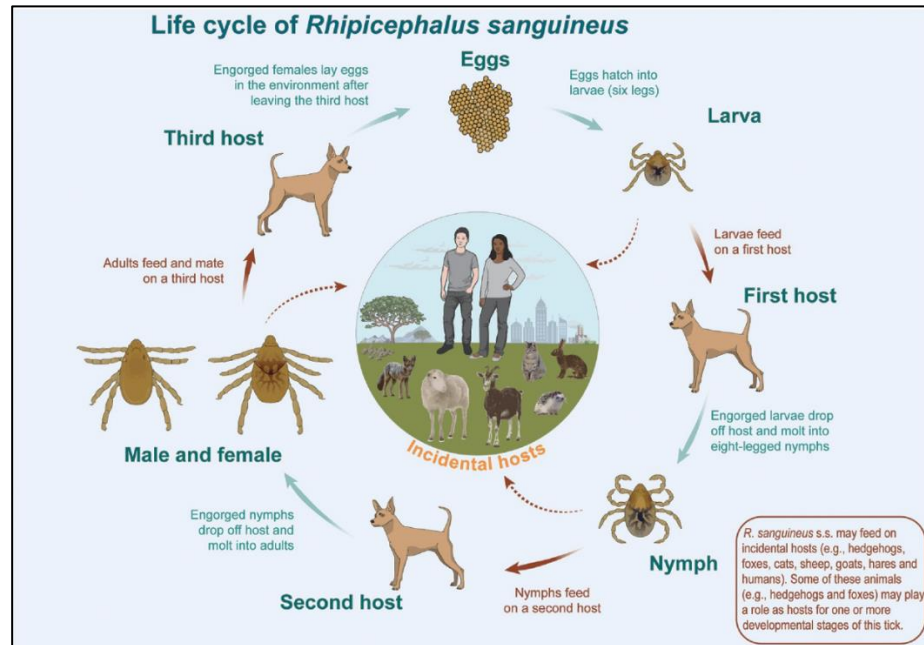


Figure 3: Life cycle of *Rhipicephalus sanguineus* (Dantas-Torres & Otranto, 2022).

2.3.2 Off-host Ecology

Most ixodid ticks are exophilic, i.e., they have a tendency to stay outdoors. Conversely, the Brown dog ticks are by nature, endophilic (accustomed to staying indoor) albeit infrequently exophilic, and monotropic, whereby the same host species is fed by all developmental stages (Dantas-Torres *et al.*, 2013). Nevertheless, Dantas-Torres (2008) refuted that the tick endophilic behaviour is otherwise in tropical areas where they are usually found to be exophilic. The off-host tick stages have a preference to hide in cracks and crevices, usually near to the host resting or sleeping areas (Dantas-Torres, 2008). In highly infested places, they can be found crawling on the ground, furniture and walls (Dantas-Torres *et al.*, 2006).

Since *R. sanguineus* spends most of the time in the environment, they are influenced by both biotic and abiotic factors. The prevalence and degree of tick infestation vary according to geographical areas and seasons (Dantas-Torres, 2010). For example, the ticks are more prevalent all year round in tropical and subtropical countries than in temperate countries. In

temperate zones, it is notable that the Brown dog ticks are more active from summer, through spring to early autumn (Dantas-Torres, 2008).

Furthermore, population density, ectoparasitic control status, lifestyle, breed and age can also be the factors. In terms of population density, Brown dog tick infestation is especially high in dog shelters and the stray dog population. When compared to pet dogs which mostly have good ectoparasitic control, stray dogs and rural dogs tend to have higher tick prevalence as concluded by Dantas-Torres *et al.* (2009). Apart from that, certain breeds such as English Cocker Spaniel dogs are more predisposed to *R. sanguineus* infestation in comparison to mongrel dogs (Dantas-Torres *et al.*, 2006). In addition, young dogs have higher degree of tick infestation (Dantas-Torres, 2010).

2.4 Tick Saliva and The Susceptibility of Dogs to *R. sanguineus* Infestations

The main organs responsible for osmoregulation in ticks are the salivary glands and the Malpighian tubules (Dantas-Torres, 2008). Upon attachment on the host, the ticks inoculate their saliva, which possesses strong immunomodulatory substances that enable the ticks to remain attached throughout their blood-feeding process for an extended duration (Valenzuela, 2004). It is interesting to note that the immunomodulatory substances comprising vasodilators, antihaemostatic, anti-inflammatory, anaesthetics and immunosuppressive molecules, are known to suppress the host immune and inflammatory response.

In addition, Stibrániová *et al.* (2013) stipulated that the immunomodulatory effects also facilitate and increase the transmission risk of tick-borne pathogens. It has been demonstrated that the Brown dog tick saliva impedes T-cell proliferation and macrophages' microbicidal activity. Cavassani *et al.* (2005) hypothesised that the differentiation and maturation of dendritic cells into functional antigen-presenting cells (APCs) are inhibited by the tick saliva. This suggests that dogs exposed to repeated *R. sanguineus* infestations fail to develop a delayed-type hypersensitivity (DTH) response, indicating a poor cell-mediated immunity. Hence, this renders

the dogs with no resistance to *R. sanguineus* reinfestations as the *R. sanguineus* exhibits evolved salivary immunomodulatory factors to modulate the immunological response to their advantage.

2.5 Veterinary and Medical Significance

Aziz *et al.* (2017) stated that the blood-sucking activities of *R. sanguineus* cause detrimental effects to the dogs such as skin irritation and damage. It is also notable in the transmission of several tick-borne diseases such as canine ehrlichiosis (*Ehrlichia canis*, *E. chaffeensis*), anaplasmosis (*Anaplasma phagocytophilum*), hepatozoonosis (*Hepatozoon canis*) and babesiosis (*Babesia canis*, *B. vogeli*) (Dantas-Torres *et al.*, 2013; Sosa-Gutierrez *et al.*, 2016). In the absence of dog hosts, Brown dog ticks conveniently parasitise humans, elevating the potential risk to contract zoonotic diseases. (Dantas-Torres, 2010). *Rhipicephalus sanguineus* bites have been identified to cause pathogenic tick-borne rickettsial zoonoses (Rocky Mountain spotted fever) in Northeastern Mexico (Salomon *et al.*, 2022).

Based on Estrada-Peña *et al.* (2013), the inherent ability of ticks make them a suitable vector in the transmission of pathogens. Ticks become infected when they feed on host blood, and then they are able to transfer the pathogens trans-stadially via molting. The pathogen-exposed, molted stages, too, can transmit the pathogens to naive hosts during blood meal.

2.6 Tick Control

All the motile stages (larva, nymph and adult) can be found predominantly in the environment or transiently on the dogs during blood meals (Foreyt, 2001). Therefore, an effective tick control should take into account both the canine population and the environment.

2.6.1 Chemical Control

Tick infestation can be controlled with a diverse range of veterinary commercial products such as shampoos, dips, powders, sprays, impregnated collars and oral medication. The most

frequently used acaricides are fipronil (Frontline® spray), afoxolaner (Nexgard®) and amitraz. As approximately 95% of the ticks are off-host, the use of acaricides to treat the environment in which the dog resides is necessary (Dantas-Torres, 2008). However, excessive, long-term and indiscriminate application of chemical acaricides has led to high residue levels, environmental pollution, tick resistance and host intoxications, which includes humans (Denardi *et al.*, 2010).

2.6.2 Non-chemical Control

Non-chemical strategies should be integrated for effective tick elimination. A change in habitat can be implemented. Dantas-Torres (2008) suggested that weeds should be frequently trimmed short, and cracks and crevices should be sealed. Client education is vital by advising the dog owners to frequently examine and eliminate ticks from their dogs (Dantas-Torres *et al.*, 2006).

Biological control of ticks is also feasible. For example, a research by Kirkland *et al.* (2004) has shown that the use of entomopathogenic fungi like *Beauveria bassiana* and *Metarhizium anisopliae* may potentially control *R. sanguineus* populations. Another alternative approach is the use of traditional herbs which are typically environmentally safe, cheap and easy to apply. A study by Denardi *et al.* (2010) has proven that the use of neem (*Azadirachta indica*) at 10% and 20% caused significant changes in the reproductive system of Brown dog ticks such as fragmentation of nucleoli in the germinal vesicle of an engorged female tick.

2.7 *Nicotiana tabacum*

Tobacco (*Nicotiana tabacum*) is a perennial herbaceous plant. Tobacco is commercially cultivated to be processed into tobacco and has been beneficial in the traditional medicine (Agyare *et al.*, 2013). It was claimed to exhibit parasitic control such as tick and mange control in dogs, treatment of maggot wounds, leech and insect repellent for humans and agricultural pest control (R. Michael, personal communication, December 26, 2022). According to Weber *et al.* (2019), *N.*

tabacum is an effective pesticide and used as an insecticide or acaricide to prevent vector-borne diseases.

It is widely used in Sabah due to its low cost, local production and social acceptance (Chong, 1995). *Nicotiana tabacum* contains nicotine as the major alkaloid which exhibits insecticidal and acaricidal effects. Nicotine acts as an agonist for the nicotine acetylcholine (nAch) receptor and works by overstimulating the insect's nervous system, leading to intensive tremors, convulsions and eventual paralysis (Madhukar *et al.*, 2012). According to a study by Akanda *et al.* (2015), the effectiveness of tobacco against tick infestation was 60% at 1% concentration.

2.8 Fipronil

Fipronil is the active ingredient in Frontline® spray, a type of chemical acaricide used in small animals. It is classified under selective GABA (gamma-aminobutyric acid) antagonist because it inhibits the passage of chloride ions through GABA-gated chloride channels by binding to the GABA-receptors (Castro-Janer *et al.*, 2009; Food and Agriculture Organization, 2009). Cole *et al.* (1993) mentioned that in ticks, fipronil can cause mortality at high doses, whereas low concentrations may lead to nervous function disruption. Fipronil is effective for tick control, but it can be toxic to non-target organisms including mammals, resulting in toxicity in nerve cells, hepatocytes, nephrons, reproductive and endocrine cells (Abdel-Daim *et al.*, 2019; Obaid *et al.*, 2022).

3.0 MATERIALS AND METHODS

3.1 Study Animals

A total of 240 adult stages of Brown dog ticks comprising of 120 females and 120 males were collected from the environment of the shelter dogs at Malaysia Independent Animal Rescue (MIAR), Semenyih, Selangor using forceps. The ticks were morphologically identified as *Rhipicephalus sanguineus* using the morphological keys as described by González-Álvarez *et al.* (2020), segregated according to gender and kept in 50mL falcon tubes as specimen containers covered with a filter paper with multiple needle-sized holes for ventilation and maintained in the room temperature until further use. The ticks were used within 48 hours post-collection.

3.2 Preparation of Tobacco Aqueous Extract

Dried leaves of *Nicotiana tabacum* were procured from a local market in Penampang, Sabah. The tobacco aqueous extract was prepared by maceration and digestion method (Anshu *et al.*, 2020). Approximately 25 grams of dried tobacco leaves was ground into fine powder using a pestle and mortar. The dried leaf powder was placed in a conical flask and 100mL of sterile distilled water was added to make 100% stock solution (250mg/mL). The flask was then heated in 60°C water bath for five minutes. The flask was placed in room temperature for 24 hours with frequent mixing to liberate the active ingredients. The extract was strained with a small tea strainer of mesh sizes less than 1mm and subsequently passed through a 70mm filter paper. The filtered extract was stored at 4°C as a stock solution until further use.

3.3 Dilution Preparation

The stock solution (250mg/mL as 100%) of tobacco aqueous extract was diluted sequentially by half of the previous concentration. The dilutions were 50% (125mg/mL), 25% (62.5mg/mL), 12.5% (31.25mg/mL) and 6.25% (15.625mg/mL). Each of the dilutions was prepared by taking the calculated volume from the stock solution and then adding sterile distilled water to make up a total

of 20mL per concentration in 100mL Scott bottles. The total volume required for both tobacco aqueous extract and sterile distilled water were calculated using the formula:

$$M_1V_1 = M_2V_2$$

Whereby, M = Molarity ; V = Volume

3.4 In vitro Acaricidal Efficacy of Tobacco Aqueous Extract and Fipronil

A total of seven groups of adult ticks (N = 10) with each 5 females and 5 males were prepared to observe the acaricidal efficacy. The first five groups were treated with 100%, 50%, 25%, 12.5% and 6.25% concentration of tobacco aqueous extract; the sixth group with 0.29% fipronil (Frontline® spray) as a positive control; the seventh group as a negative control (sterile distilled water). Each group had four replicates to make up 20 male ticks and 20 female ticks per treatment group (N = 40) (Adenubi *et al.*, 2018).

3.4.1 Experiment 1: Envelope Test

A pipette was used to apply 0.5mL of each solution onto a 70mm filter paper placed in a petri dish. Five male and five female adult ticks were transferred onto each of the filter papers using soft forceps. A second filter paper was placed on top of the first one, and another 0.5mL of the corresponding solution was dropped onto the second filter paper, so that the filter papers sandwiched the ticks. The tick mortality was observed at exponential time interval of 0 min, 1 min, 3 mins, 6 mins, 12 mins, 24 mins, 48 mins, 1.5 hours, 3 hours, 6 hours, 12 hours and 24 hours. The ticks' movement was provoked by pricking them with sharp-tipped forceps under a dissecting microscope to confirm their mortality. The experiment was repeated for all the treatment groups and replicates.

3.4.2 Experiment 2: Immersion Test

Ten milliliters of each solution of the seven treatment groups were poured into 50mL beakers. With the aid of soft forceps, five male and five female adult ticks were immersed in the respective solutions for less than 5 seconds and then swiftly placed onto a 70mm filter paper in a petri dish. The observation method was performed using the same method described in Experiment 1.

Table 1: The observation table for both Experiment 1 and 2 to determine the *in vitro* acaricidal efficacy between tobacco aqueous extract and fipronil on the Brown dog ticks.

Interval	Tick Mortality (Survived: _/5)														Remarks								
	Time	Negative control		Time	Fipronil		Time	100%		Time	50%		Time	25%		Time	12.5%		Time	6.25%			
		M	F		M	F		M	F		M	F		M		F		M	F		M	F	
0 min																							
1 min																							
3 mins																							
6 mins																							
12 mins																							
24 mins																							
48 mins																							
1.5 hrs																							
3 hrs																							
6 hrs																							
12 hrs																							
24 hrs																							

3.5 Data Analysis

The data collected were analysed with Microsoft Excel 2016 and IBM SPSS Statistics Software 27. Using Microsoft Excel 2016, the mortality percentage (%) was calculated, and bar charts and line graphs were plotted. The formula is as follows:

$$\frac{\text{Number of dead ticks}}{\text{Initial total number of ticks}} \times 100\%$$

Using SPSS, the data was tested for normality (Kolmogorov-Smirnov test). The data were not normally distributed, hence descriptive statistics were analysed using Kruskal-Wallis test along with Post-Hoc test for pairwise comparison. Lethal Dose 50 (LD_{50}) and Lethal Time 50 (LT_{50}) values were calculated via Probit analysis (Pillai *et al.*, 2021). The statistical significance was set at $P = 0.05$ with confidence interval of 95%.



4.0 RESULTS

4.1 *In vitro* Acaricidal Efficacy of Different Treatment Groups

4.1.1 Experiment 1: Envelope Test

A total of 40 ticks were used in each of the treatment group. At the end of the experiment (after 24 hours), all the ticks died for the treatment group fipronil, and 100% (250mg/mL), 50% (125mg/mL), 25% (62.5 mg/mL) and 12.5% (31.25 mg/mL) concentrations of tobacco (*N. tabacum*) aqueous extract as represented by the bar chart (Figure 4). However, four female ticks remained alive in 6.25% (15.625 mg/mL) concentration of tobacco aqueous extract. The result was valid as none of the ticks died in the negative control group.

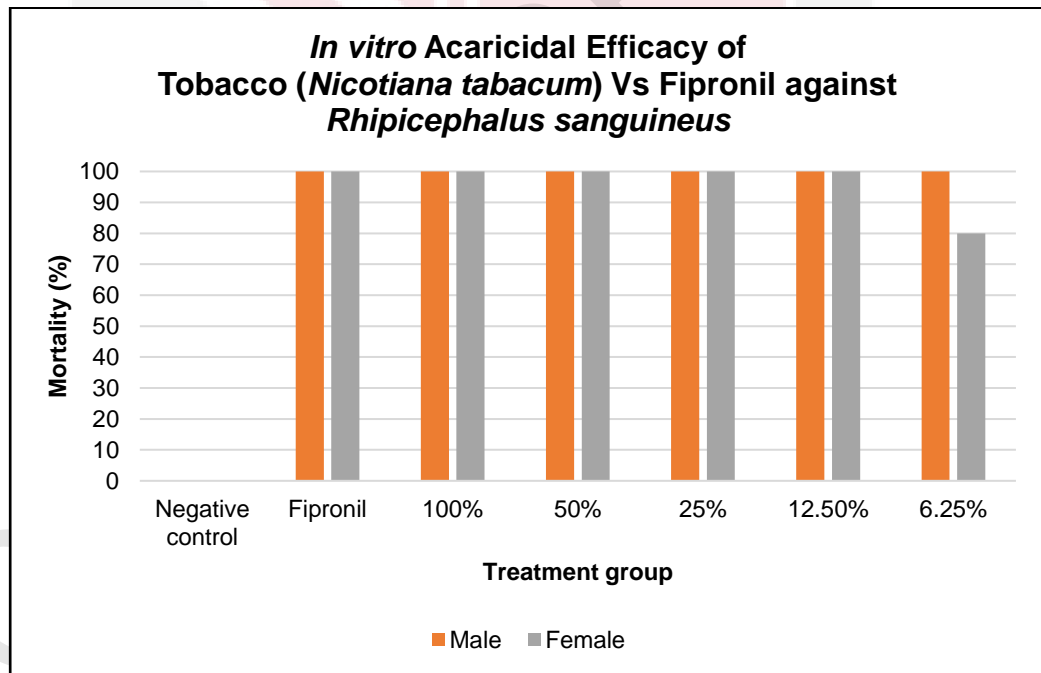


Figure 4: Bar chart showing the percentage of the dead tick population at the end of Experiment 1 (Envelope Test) after 24 hours.

After 24 hours, based on the descriptive analysis in Table 2, the mean rank was equal for fipronil and all of tobacco aqueous extract concentrations, except for 6.25% extract which was

25.00. As for the negative control, it was 4.50 only. The negative control group showed significant difference at $P < 0.05$ across all the other treatment groups. However, there were no significant difference between fipronil and all the concentrations of tobacco aqueous extract as illustrated in Table 3.

Table 2: Descriptive statistics of Envelope test using Kruskal-Wallis analysis after 24 hours.

	Treatment group	Mean rank
Number of Dead Ticks	Negative control	4.50
	Fipronil	34.00
	100%	34.00
	50%	34.00
	25%	34.00
	12.5%	34.00
	6.25%	25.00

Table 3: Significant differences between treatment groups in Envelope test after 24 hours.

Treatment group	Fipronil	100%	50%	25%	12.50%	6.25%
Negative control	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
Fipronil		1	1	1	1	0.111
100%			1	1	1	0.111
50%				1	1	0.111
25%					1	0.111
12.50%						0.111

4.1.2 Experiment 2: Immersion Test

A total of 40 ticks were used in this experiment for each treatment group. No mortality of ticks was observed in the negative control group after 24 hours. At the end of the experiment, 90% of female ticks and 90% of male ticks died after exposure to fipronil and 100% concentration of tobacco aqueous extract respectively. The mortality percentage recorded at 60% male ticks and

65% female ticks for the aforementioned treatment groups respectively. As the concentration of the tobacco aqueous extract reduced, the trend demonstrated a dwindling tick population with the female ticks appeared to be of higher survivability than the male ticks after exposure to the treatment groups. The following bar chart (Figure 5) shows the outcome of this experiment.

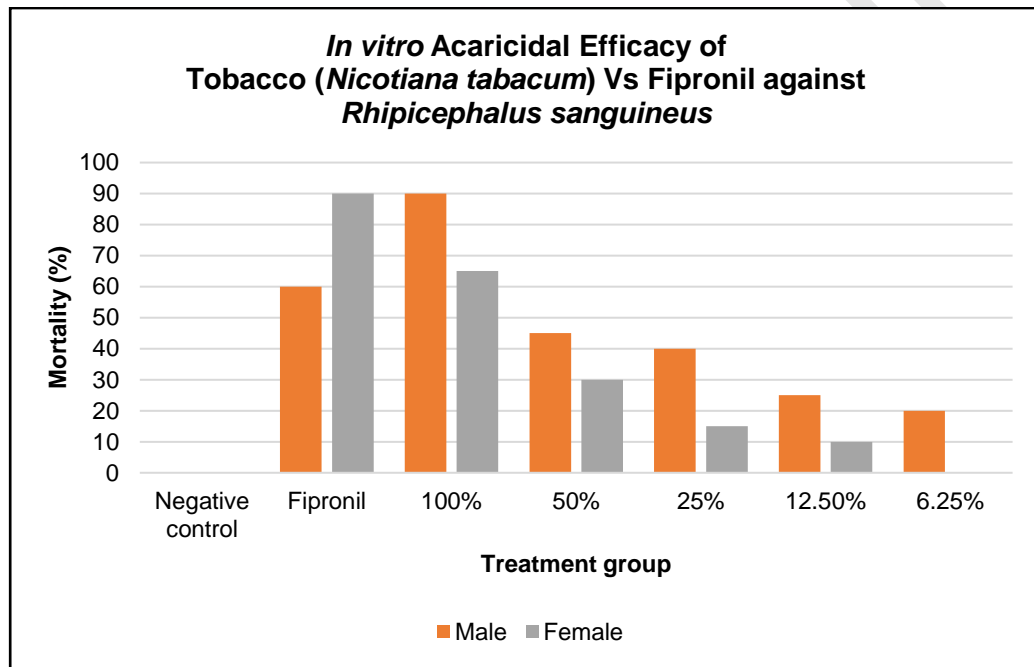


Figure 5: Bar chart showing the percentage of the dead tick population at the end of Experiment 2 (Immersion Test) after 24 hours.

As tabulated in Table 4, the descriptive analysis demonstrated a decrease in mean rank as the tobacco aqueous extract decreased. The 100% tobacco extract recorded a higher mean rank than fipronil with the negative control having the lowest mean rank. As depicted in Table 5, only fipronil, 100%, 50% and 25% tobacco aqueous extract concentrations had statistical differences against the negative control. 100% extract concentration showed no significant difference with fipronil. Extract concentrations of 25% and below were observed to be significantly different than

fipronil and 100% extract concentration. 50% extract concentration was statistically different than the 100% but not fipronil. The remaining of the treatment groups had no significant difference.

Table 4: Descriptive statistics of Immersion test using Kruskal-Wallis analysis after 24 hours.

	Treatment group	Mean rank
Number of Dead Ticks	Negative control	11.00
	Fipronil	45.69
	100%	46.50
	50%	30.56
	25%	26.94
	12.5%	21.19
	6.25%	17.63

Table 5: Significant differences between treatment groups in Immersion test after 24 hours.

Treatment group	Fipronil	100%	50%	25%	12.50%	6.25%
Negative control	< 0.001	< 0.001	0.013	0.043	0.197	0.401
Fipronil		0.918	0.055	0.018	0.002	< 0.001
100%			0.043	0.013	0.001	< 0.001
50%				0.646	0.235	0.101
25%					0.466	0.238
12.50%						0.652

4.2 *In vitro* Acaricidal Efficacy of Different Treatment Groups By Gender Across Time

4.2.1 Experiment 1: Envelope Test

The 100% tobacco extract concentration portrayed the highest acaricidal efficacy as all the ticks died after 48 minutes. The other lower extract concentrations came after with fipronil being more efficacious than the lowest extract concentration (6.25%). To kill all the ticks, fipronil required a total of 24 hours. A 100% mortality was achieved at the 6th hour for 50% and 25% tobacco extract concentrations, meanwhile the other groups after half a day.

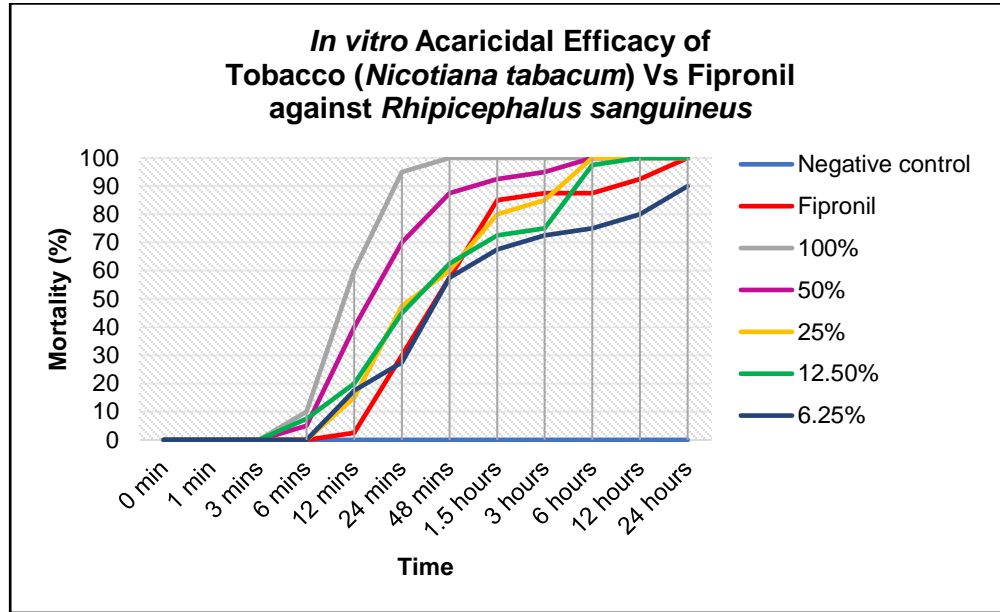


Figure 6: Line graph showing the total tick population mortality rate across treatment groups in Envelope test.

Referring to Table 6, LT_{50} of 100% tobacco extract was almost 11 mins. As the concentration decreased, the LT_{50} lengthened. On the other hand, LT_{50} of fipronil was recorded at almost 50 mins, almost five times longer than that of 100%. The acaricidal effects of at least 12.5% extract was proven to be better than fipronil.

Table 6: Lethal Time 50 (LT_{50}) of the treatment group in Envelope test

Time	Fipronil	100%	50%	25%	12.50%	6.25%
Minutes	49.841	10.834	18.747	37.771	39.305	76.588
Minimum	34.106	9.465	13.676	30.944	31.250	58.255
Maximum	70.135	12.391	25.228	45.932	49.239	100.860

4.2.1.1 Mortality of Male *Rhipicephalus sanguineus*

Figure 7 represents the relationship between time and male tick mortality across all the treatment groups. The line graph indicated that fipronil and all the concentrations of tobacco

aqueous extract displayed some acute toxicity effect on the ticks because the mortality was recorded as early as 6 to 24 mins, especially in the higher extract concentrations. The tick mortality reached its peak (100% mortality) first in the 100% extract concentration, followed by 50%, 25%, 12.5% and 6.25% extract concentrations, and finally fipronil, sequentially. The male ticks exposed to fipronil died at a slower rate compared to the rest. All the ticks eventually died except for the negative control, which recorded zero mortality.

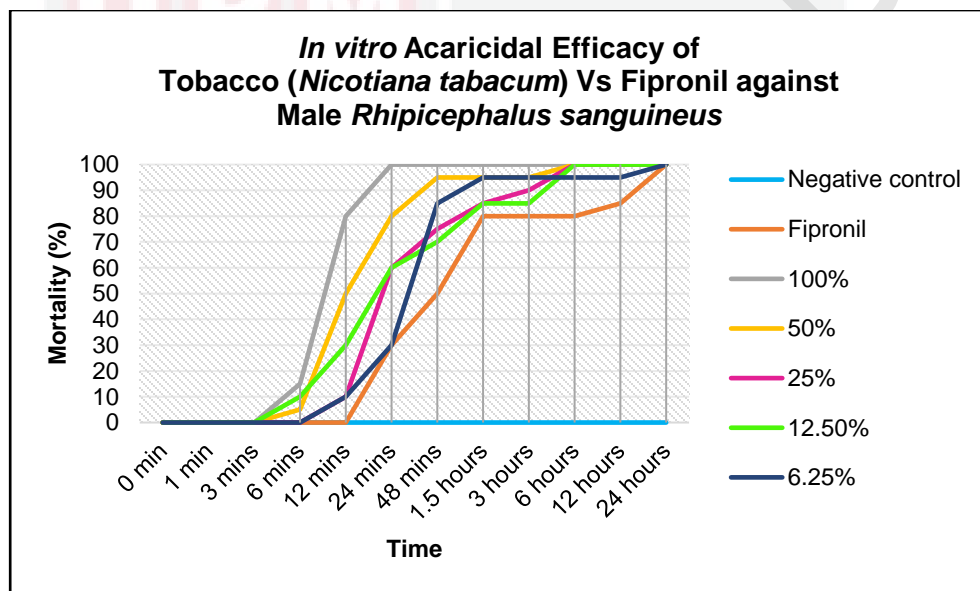


Figure 7: Line graph showing the male tick population mortality rate across treatment groups in Envelope Test.

4.2.1.2 Mortality of Female *Rhipicephalus sanguineus*

Figure 8 depicts the relationship between time and female tick mortality across all the treatment groups. The graph described the eventual mortality of all female ticks for all treatment groups except for the 6.25% tobacco extract concentration and the negative control groups. At 100% extract concentration, the female tick mortality spiked up dramatically from the 6th min to the 48th min in comparison to other treatment groups. The climb towards 100% mortality was followed by 50% and 25% extract concentrations, then fipronil and 12.5% extract concentration.

20% of the female ticks survived the 6.25% extract concentration after 24 hours. The female ticks exposed to fipronil died at a higher rate as opposed to that of less than 50% extract concentrations.

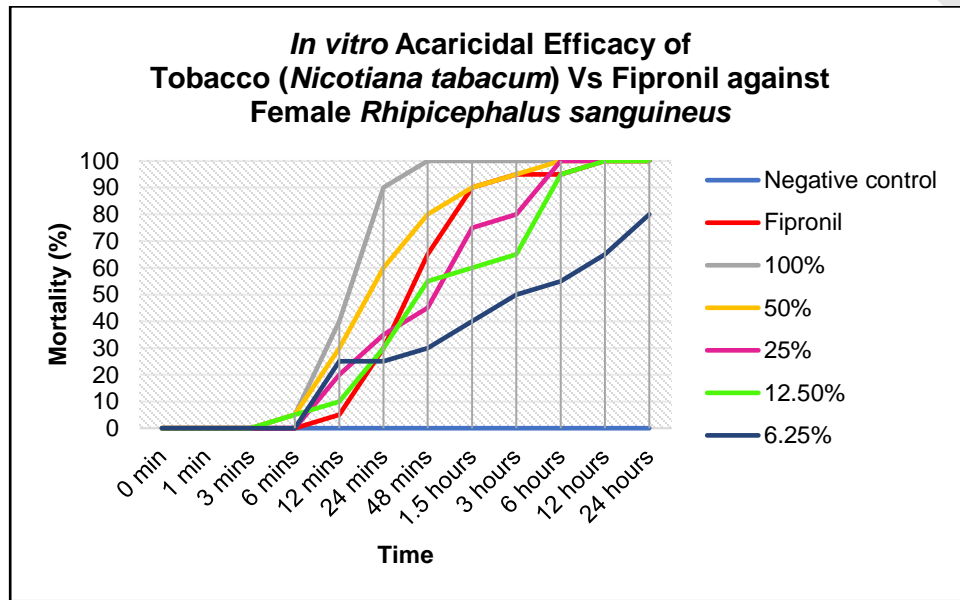


Figure 8: Line graph showing the female tick population mortality rate across treatment groups in Envelope Test.

4.2.2 Experiment 2: Immersion Test

Among all treatment groups, 100% tobacco extract concentration showed a consistent and gradual acaricidal effects overtime, killing almost 80% of the tick population. The residual effect in fipronil set in effectively within 1.5 hours and gradually caught up with the ultimate mortality rate in 100% extract concentration. The acaricidal effects of fipronil was inferior to 100% extract. 50% extract concentration plateaued just below 40% mortality. The other lower concentrations barely killed more than 30% of the total tick population.

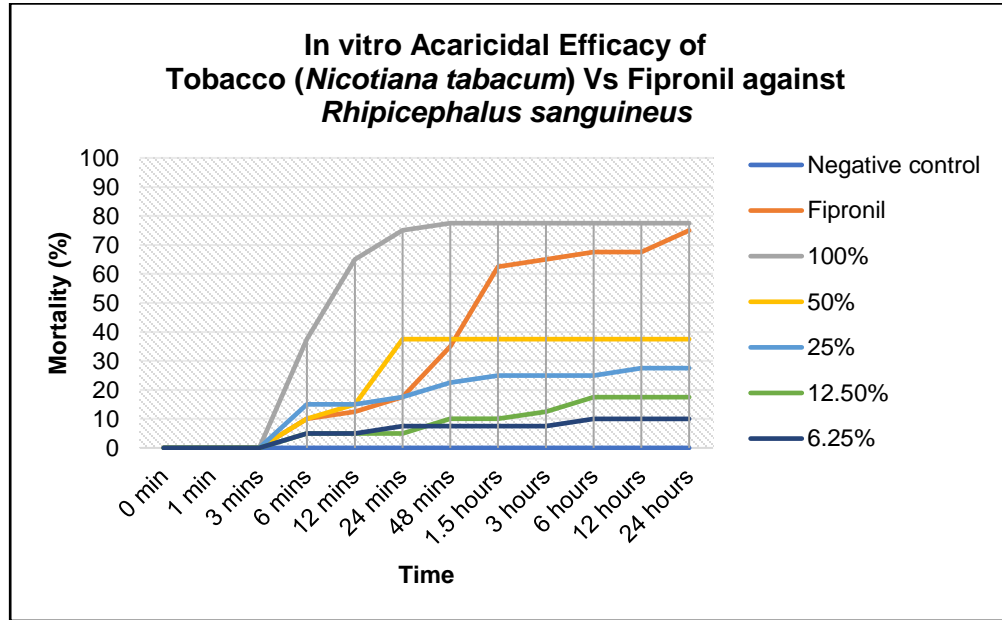


Figure 9: Line graph showing the total tick population mortality rate across treatment groups in Immersion Test.

As shown in Table 7, the acaricidal effect of 100% tobacco extract concentration was superior to fipronil as it only took barely 22 mins to kill off 50% of the tick population. LT_{50} of the subsequent lower doses was almost or at least a day, which was way longer than that of fipronil which was approximately 2.4 hours.

Table 7: Lethal Time 50 (LT_{50}) of the treatment group in Immersion test.

Time	Fipronil	100%	50%	25%	12.5%	6.25%
Minutes	141.395	21.56	1280.724	12337.248	67056.655	651 3237.827
Minimum	102.742	10.282	357.489	1929.562	8476.482	62079.493
Maximum	200.162	39.722	31707.544	201 9408.492	1212 9643.23	4.778E+17

4.2.2.1 Mortality of Male *Rhipicephalus sanguineus*

Based on Figure 10, it was observed that none of the treatment groups had 100% mortality for Immersion test. Among all the treatment groups, only 100% tobacco extract concentration

exhibited the highest acaricidal efficacy which was at 90% mortality of male ticks and plateaued after 48 mins. The acaricidal effect of fipronil surged at a maximum of 60% mortality. Descendingly, the mortality rate decreased to below 50% for the male ticks dipped in 50%, 25%, 12.5% and 6.25% of extract concentrations. The negative control presented a valid result.

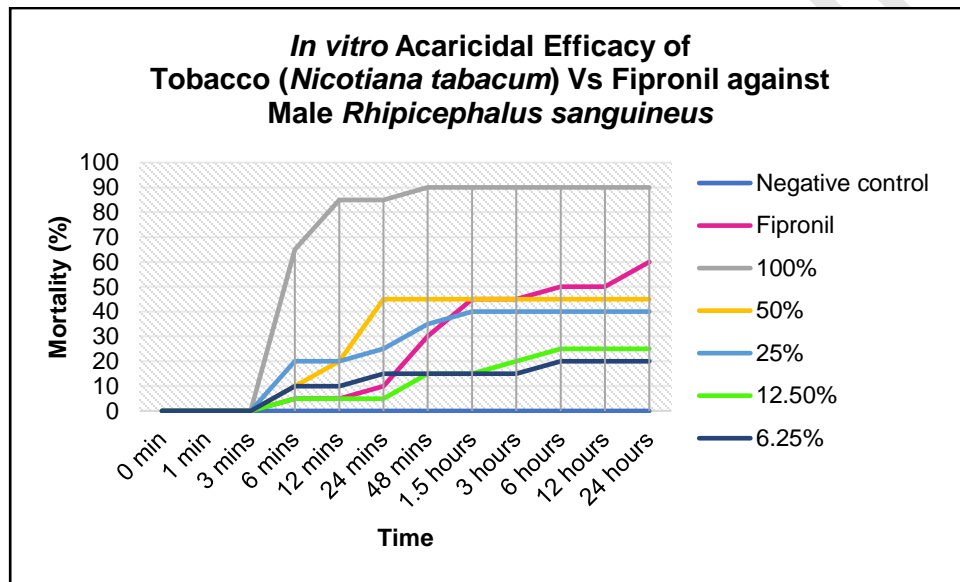


Figure 10: Line graph showing the male tick population mortality rate across treatment groups in Immersion Test.

4.2.2.2 Mortality of Female *Rhipicephalus sanguineus*

The illustration in Figure 11 also highlighted a relatively lower acaricidal efficacy of tobacco aqueous extracts for the concentrations of 50% and below. Fipronil had the highest efficacy reported at 90% mortality, with 100% tobacco extract concentration came the second at 65% mortality. 6.25% extract concentration was proven to be ineffective against the female ticks as none of them died. No mortality was observed in the negative control group.

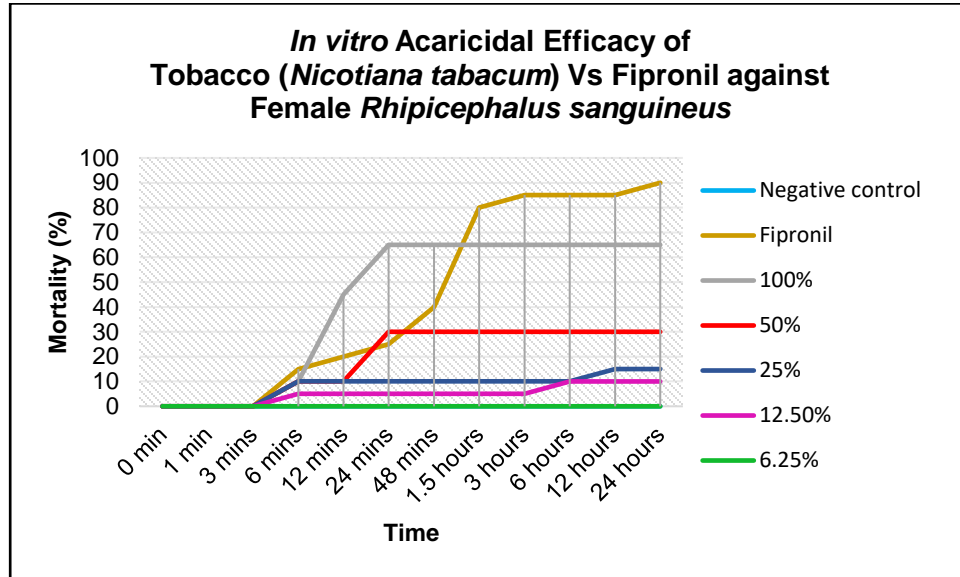


Figure 11: Line graph showing the female tick population mortality rate across treatment groups in Immersion Test.

4.3 Comparison of *In vitro* Acaricidal Efficacy Between Envelope Test and Immersion Test

Based on the presented data, after 24 hours, Experiment 1 (Envelope test) resulted in higher acaricidal efficacy than Experiment 2 (Immersion test) against *R. sanguineus* as most of the ticks died at the end of Experiment 1. After Kruskal-Wallis analysis was performed between the two experiments, the acaricidal efficacy against the ticks had no significant difference among the negative control, fipronil and 100% extract concentration. However, the rest of the tobacco aqueous extract concentrations produced a significant difference as shown in Table 8.

Table 8: Significant values of the *in vitro* acaricidal efficacy of the treatment groups between Envelope test and Immersion test after 24 hours.

Experiment	Treatment group						
	Negative control	Fipronil	100%	50%	25%	12.50%	6.25%
Significant values	1	0.088	0.142	0.003	<0.001	<0.001	<0.001

4.3.1 Comparison of Mortality of *Rhipicephalus sanguineus* Between Genders

For Envelope test, by comparing both Figure 7 and Figure 8, it was noticed that all the tobacco aqueous extract concentrations were relatively more potent to the male ticks than the female ticks. In contrast, upon exposure to fipronil, the male ticks survived for a longer duration than the female ticks before their eventual death. Despite the differences in mortality rate, only 6.25% extract concentration of all the treatment groups concluded a significant difference between the genders as tabulated in Table 9.

For Immersion test, referring to Figure 10 and Figure 11, it is clear that the male ticks were potentially more resistant to fipronil than the female ticks. Conversely, the male ticks were more susceptible to 100% tobacco extract concentration than the opposite gender. These inferences were further supported by Kruskal-Wallis analysis with a significant difference between genders for ticks treated with fipronil and 100% extract concentration. 25% and 6.25% extract concentrations also yielded similar results.

Table 9: Significant values of the *in vitro* acaracidal efficacy of the treatment groups between genders for both experiments.

Experiment	Treatment group						
	Negative control	Fipronil	100%	50%	25%	12.5%	6.25%
Envelope Test	1	0.315	0.384	0.516	0.314	0.163	0.003
Immersion Test	1	0.001	<0.001	0.226	<0.001	0.236	<0.001

4.3.2 Comparison of Lethal Time 50 (LT₅₀)

At the end of the experiment, the LT₅₀ of Envelope test was shorter than that of Immersion test as illustrated in Table 10, making Envelope test a superior choice of treatment method.

Table 10: Lethal Time (LT₅₀) between Envelope test and Immersion test across treatment groups in minutes.

Experiment	Fipronil	100%	50%	25%	12.5%	6.25%
Envelope Test	49.841	10.834	18.747	37.771	39.305	76.588
Immersion Test	141.395	21.56	1280.724	12337.248	67056.655	651 3237.827

4.3.3 Comparison of Lethal Dose 50 (LD₅₀)

After 24 hours, the LD₅₀ of Envelope test was 5.477mg/mL concentration of tobacco aqueous extract, whereas for Immersion test, it was 128.928mg/mL, which was remarkably higher than the former as shown in Table 11.

Table 11: Lethal Dose 50 (LD₅₀) of tobacco aqueous extract on *R. sanguineus* after 24 hours.

Experiment	Concentration of Tobacco Aqueous Extract (mg/mL)
Envelope Test	5.477
Immersion Test	128.928

5.0 DISCUSSION

The Envelope test showed that all the treatments possessed certain acaricidal effects against *R. sanguineus* with almost 100% mortality in all groups at the end of the experiment. The acaricidal efficacy in all concentrations of tobacco aqueous extract were comparable to fipronil. Jeyathilakan *et al.* (2019) had observed that tobacco aqueous extract caused impaired movements and absence of pedal reflex in adult female *R. sanguineus* after 15 mins of 100% and 50% extract application. The same paralytic effects using *N. tabacum* against *Rhipicephalus haemophysaloides* were also reported by Choudhary *et al.* (2004).

In Immersion test, it was shown that 12.5% and 6.25% tobacco aqueous extract concentrations barely had acaricidal effects. It can also be said that only 100% and 50% extract were comparable to fipronil. The acaricidal effect of the tobacco extracty was concentration and time dependent. Anshu *et al.* (2020) presented similar findings of dose and time dependent mortality using *N. tabacum* against *Rhipicephalus (Boophilus) microplus*.

The 100% (250mg/mL) extract had the best acaricidal effects as the LT₅₀ was the shortest. Lethal time 50 (LT₅₀) was described as the total time required for a treatment drug to induce the death of 50% of the animals (Nguyen *et al.*, 2016). The same authors also found that LT₅₀ of 20% (200mg/mL) and 2% tobacco extract (*Nicotiana rustica* L.) were 35 mins and 70 mins respectively to kill dog ticks with no side effects. The LT₅₀ was concentration dependent, similar to that reported by Nguyen *et al.* (2016) which concluded that following the decrease in tobacco extract concentrations, there were increase in LT₅₀ values induced by extracts to dog ticks.

All in all, tobacco aqueous extract was comparable to fipronil. Based on LT₅₀, at least 12.5% extract concentration in Envelope test and 100% extract concentration in Immersion test were superior to the acaricidal efficacy of fipronil. In this experiment, most of the ticks subjected to fipronil died eventually, implying the absence of fipronil resistance. This was evidenced in an investigation by Katalbas *et al.* (2020) demonstrating 91.6% to 100% *R. sanguineus* larval mortality was observed in all fipronil doses and commercial products via larval packet test.

Since both fipronil and tobacco aqueous extract were somewhat equally potent as an acaricide, cost must be taken into account to decide on the best potential acaricide. To acquire a litre of tobacco aqueous extract, 250g was required to make 100% extract (250mg/mL) as in this experiment. It cost about RM125 (100g = RM50). As for fipronil, 1 litre cost approximately RM510 (100mL = RM51). Hence, tobacco aqueous extract would be more cost effective and should be considered as a replacement of the non-environment-friendly chemical acaricide, fipronil.

Through this experiment, Envelope test was found to exhibit better acaricidal efficacy than Immersion test as all the concentrations of tobacco aqueous extract and fipronil successfully killed all *R. sanguineus*. This can be attributed to the constant and prolonged exposure of Envelope test of a maximum of 24 hours in comparison to Immersion test which only lasted for less than 5 seconds (one-time exposure). The residual effect on the ticks in Immersion test might be low, resulting in a lower mortality rate. Kemal *et al.* (2020) found that only 77% of adult *R. sanguineus* died at 24-hour post-exposure period of 2-min immersion. Another study by Oyagbemi *et al.* (2019) described that packet test with N-hexane leaf extract of *N. tabacum* yielded higher *R. sanguineus* mortality of 99.3% compared to immersion test at 4.33% - 9.33% only.

An additional evidence through LD₅₀ further sustained the superior acaricidal effects in Envelope test because the required concentration of tobacco aqueous extract was almost half of that required in Immersion test. Lethal Dose 50 (LD₅₀) refers to the dose of a substance that kills 50% of animals under the influence of the dose used to determine the acute toxicity of drugs and pesticides (Pillai *et al.*, 2021). The lower the LD₅₀, the more toxic the solution is.

In terms of gender difference, genders might not play a role here because the significant differences across the treatment groups were inconsistent. This could be due to the absence of engorged ticks during tick collection and a sample size that was too small to justify the gender role. Future studies should consider determining the bioactive substance(s) in tobacco (*N. tabacum*), investigate the acaricidal effects across all developmental stages of the tick, and to study any potential side effects to the host.

6.0 CONCLUSION

The aqueous extract of *N. tabacum* from Sabah, Malaysia, exhibited the potential to be utilised as an acaricide against adult *R. sanguineus* by killing all the ticks after 48 minutes of exposure to the 100% concentration, with the shortest LT_{50} value of 10 minutes in Envelope test. Its acaricidal efficacy was concentration-dependent and comparable to fipronil based on the mortality percentage after 24 hours and LT_{50} values. It was noted that Envelope test was superior to Immersion test, as both the LT_{50} and LD_{50} of the former were of lower values. Interestingly, tobacco was proven to be much cheaper than fipronil by four times rendering tobacco more cost-effective. Hence, the local community can consider the use of tobacco aqueous extract as an effective and environmental friendly acaricide against the adult Brown dog ticks in the environment.

To improve the experimental outcome, further research is required to determine the bioactive substance(s) of tobacco, the effects of tobacco on all developmental stages of ticks and the side effects on dogs, so that tobacco may potentially emerge as a replacement for the commercial acaricide.

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8.0 APPENDICES

Table i: LD₅₀ of Envelope test and Immersion test by different time intervals

Time	LD ₅₀ (mg/mL)	
	Envelope test	Immersion test
12 mins	221.075	234.085
24 mins	44.813	149.72
48 mins	30.561	137.952
1.5 hrs	16.905	135.462
3 hours	13.399	134.154
6 hours	10.235	128.928
12 hours	9.448	128.928
24 hours	5.477	128.928

Table ii: Experiment 1: Acaricidal effects between tobacco (*N. tabacum*) aqueous extract and fipronil against Brown dog ticks (*R. sanguineus*).

Time	Tick Status	Negative control	Fipronil	Concentrations of tobacco (<i>N. tabacum</i>) aqueous extract				
				100%	50%	25%	12.5%	6.25%
0 min	Male	0	0	0	0	0	0	0
	Mortality (%)	0	0	0	0	0	0	0
	Female	0	0	0	0	0	0	0
	Mortality (%)	0	0	0	0	0	0	0
	Total	0	0	0	0	0	0	0
	Mortality (%)	0	0	0	0	0	0	0
1 min	Male	0	0	0	0	0	0	0
	Mortality (%)	0	0	0	0	0	0	0
	Female	0	0	0	0	0	0	0
	Mortality (%)	0	0	0	0	0	0	0
	Total	0	0	0	0	0	0	0
	Mortality (%)	0	0	0	0	0	0	0
3 mins	Male	0	0	0	0	0	0	0
	Mortality (%)	0	0	0	0	0	0	0
	Female	0	0	0	0	0	0	0
	Mortality (%)	0	0	0	0	0	0	0
	Total	0	0	0	0	0	0	0
	Mortality (%)	0	0	0	0	0	0	0
6 mins	Male	0	0	3	1	0	2	0
	Mortality (%)	0	0	15	5	0	10	0
	Female	0	0	1	1	0	1	0
	Mortality (%)	0	0	5	5	0	5	0
	Total	0	0	4	2	0	3	0
	Mortality (%)	0	0	10	5	0	7.5	0
12 mins	Male	0	0	16	10	2	6	2
	Mortality (%)	0	0	80	50	10	30	10
	Female	0	1	8	6	4	2	5
	Mortality (%)	0	5	40	30	20	10	25
	Total	0	1	24	16	6	8	7
	Mortality (%)	0	2.5	60	40	15	20	17.5
24 mins	Male	0	6	20	16	12	12	6
	Mortality (%)	0	30	100	80	60	60	30
	Female	0	6	18	12	7	6	5
	Mortality (%)	0	30	90	60	35	30	25

	Total	0	12	38	28	19	18	11
	Mortality (%)	0	30	95	70	47.5	45	2.5
48 mins	Male	0	10	20	19	15	14	17
	Mortality (%)	0	50	100	95	75	70	85
	Female	0	13	20	16	9	11	6
	Mortality (%)	0	65	100	80	45	55	30
	Total	0	23	40	35	24	25	23
	Mortality (%)	0	57.5	100	87.5	60	62.5	57.5
1.5 hours	Male	0	16	20	19	17	17	19
	Mortality (%)	0	80	100	95	85	85	95
	Female	0	18	20	18	15	12	8
	Mortality (%)	0	90	100	90	75	60	40
	Total	0	34	40	37	32	29	27
	Mortality (%)	0	85	100	92.5	80	72.5	67.5
3 hours	Male	0	16	20	19	18	17	19
	Mortality (%)	0	80	100	95	90	85	95
	Female	0	19	20	19	16	13	10
	Mortality (%)	0	95	100	95	80	65	50
	Total	0	35	40	38	34	30	29
	Mortality (%)	0	87.5	100	95	85	75	72.5
6 hours	Male	0	16	20	20	20	20	19
	Mortality (%)	0	80	100	100	100	100	95
	Female	0	19	20	20	20	19	11
	Mortality (%)	0	95	100	100	100	95	55
	Total	0	35	40	40	40	39	30
	Mortality (%)	0	87.5	100	100	100	97.5	75
12 hours	Male	0	17	20	20	20	20	19
	Mortality (%)	0	85	100	100	100	100	95
	Female	0	20	20	20	20	20	13
	Mortality (%)	0	100	100	100	100	100	65
	Total	0	37	40	40	40	40	32
	Mortality (%)	0	92.5	100	100	100	100	80
24 hours	Male	0	20	20	20	20	20	20
	Mortality (%)	0	100	100	100	100	100	100
	Female	0	20	20	20	20	20	16
	Mortality (%)	0	100	100	100	100	100	80
	Total	0	40	40	40	40	40	36
	Mortality (%)	0	100	100	100	100	100	90

Table iii: Experiment 2: Acaricidal effects between tobacco (*N. tabacum*) aqueous extract and fipronil against Brown dog ticks (*R. sanguineus*).

Time	Tick Status	Negative control	Fipronil	Concentrations of tobacco (<i>N. tabacum</i>) aqueous extract				
				100%	50%	25%	12.50%	6.25%
0 min	Male	0	0	0	0	0	0	0
	Mortality (%)	0	0	0	0	0	0	0
	Female	0	0	0	0	0	0	0
	Mortality (%)	0	0	0	0	0	0	0
	Total	0	0	0	0	0	0	0
	Mortality (%)	0	0	0	0	0	0	0
1 min	Male	0	0	0	0	0	0	0
	Mortality (%)	0	0	0	0	0	0	0
	Female	0	0	0	0	0	0	0
	Mortality (%)	0	0	0	0	0	0	0
	Total	0	0	0	0	0	0	0
	Mortality (%)	0	0	0	0	0	0	0
3 mins	Male	0	0	0	0	0	0	0
	Mortality (%)	0	0	0	0	0	0	0
	Female	0	0	0	0	0	0	0
	Mortality (%)	0	0	0	0	0	0	0
	Total	0	0	0	0	0	0	0
	Mortality (%)	0	0	0	0	0	0	0
6 mins	Male	0	1	13	2	4	1	2
	Mortality (%)	0	5	65	10	20	5	10
	Female	0	3	2	2	2	1	0
	Mortality (%)	0	15	10	10	10	5	0
	Total	0	4	15	4	6	2	2
	Mortality (%)	0	10	37.5	10	15	5	5
12 mins	Male	0	1	17	4	4	1	2
	Mortality (%)	0	5	85	20	20	5	10
	Female	0	4	9	2	2	1	0
	Mortality (%)	0	20	45	10	10	5	0
	Total	0	5	26	6	6	2	2
	Mortality (%)	0	12.5	65	15	15	5	5
24 mins	Male	0	2	17	9	5	1	2
	Mortality (%)	0	10	85	45	25	5	10
	Female	0	5	13	6	2	1	0

	Mortality (%)	0	25	65	30	10	5	0
	Total	0	7	30	15	7	2	2
	Mortality (%)	0	17.5	75	37.5	17.5	5	5
48 mins	Male	0	6	18	9	7	3	3
	Mortality (%)	0	30	90	45	35	15	15
	Female	0	8	13	6	2	1	0
	Mortality (%)	0	40	65	30	10	5	0
	Total	0	14	31	15	9	4	3
	Mortality (%)	0	35	77.5	37.5	22.5	10	7.5
1.5 hours	Male	0	9	18	9	8	3	3
	Mortality (%)	0	45	90	45	40	15	15
	Female	0	16	13	6	2	1	0
	Mortality (%)	0	80	65	30	10	5	0
	Total	0	25	31	15	10	4	3
	Mortality (%)	0	62.5	77.5	37.5	25	10	7.5
3 hours	Male	0	9	18	9	8	4	3
	Mortality (%)	0	45	90	45	40	20	15
	Female	0	17	13	6	2	1	0
	Mortality (%)	0	85	65	30	10	5	0
	Total	0	26	31	15	10	5	3
	Mortality (%)	0	65	77.5	37.5	25	12.5	7.5
6 hours	Male	0	10	18	9	8	5	4
	Mortality (%)	0	50	90	45	40	25	20
	Female	0	17	13	6	2	2	0
	Mortality (%)	0	85	65	30	10	10	0
	Total	0	27	31	15	10	7	4
	Mortality (%)	0	67.5	77.5	37.5	25	17.5	10
12 hours	Male	0	10	18	9	8	5	4
	Mortality (%)	0	50	90	45	40	25	20
	Female	0	17	13	6	3	2	0
	Mortality (%)	0	85	65	30	15	10	0
	Total	0	27	31	15	11	7	4
	Mortality (%)	0	67.5	77.5	37.5	27.5	17.5	10
24 hours	Male	0	12	18	9	8	5	4
	Mortality (%)	0	60	90	45	40	25	20
	Female	0	18	13	6	3	2	0
	Mortality (%)	0	90	65	30	15	10	0
	Total	0	30	31	15	11	7	4
	Mortality (%)	0	75	77.5	37.5	27.5	17.5	10