Acaricidal activities of crude extract derived from *Annona squamosa* Linnaeus leaves against cattle tick, *Rhipicephalus microplus* Canestrini (Acari: Ixodidea)

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Abstract

Rhipicephalus microplus Canestrini (Acari: Ixodidea) or cattle tick is considered as harmful and economically important external parasite, and its infestation has affected the cattle milk and meat productions worldwide. Therefore, this study aimed to evaluate the use of crude extract derived from *Annona squamosa* Linnaeus leaves for controlling engorged female *R. microplus* adults. The hexane extract of *A. squamosa* Linn. leaves at the concentrations of 4,000, 8,000, 16,000 and 32,000 ppm was used to immerse female ticks, which then were constantly monitored for their mortality and egg-laying rates for 72 h. The crude leaf extract of *A. squamosa* Linn. was shown to induce tick mortality in a dose-dependent manner with the LC_{99} value equivalent to 11,157 ppm. The concentration of the extract at 16,000 ppm was found to be the most effective and could reduce the number of ticks for 95.00±5.00% within 48 h and 100% within 72 h. The concentration of 32,000 ppm was shown to completely suppress the oviposition of females (100%), followed by 16,000 ppm (99.71±0.28%), 8,000 ppm (95.93±2.48%), and 4,000 ppm (84.57±6.53%). The effect of storage duration on efficacy of the extracts was examined and the results showed that the extract stored for 1 day could cause significantly high rates of tick mortality (100%) than the extract stored for 90 days (80.00±8.16%). Hence, this revealed that the crude hexane extract of *A. squamosa* Linn. leaves at 16,000 ppm had the highest acaricidal activity against *R. microplus* females, and the long term storage caused the marked reduction on the efficacy of the extract. This data may be helpful for further development of *A. squamosa* Linn. leaf extracts as biological control products against *R. microplus*.

Keywords*: Rhipicephalus microplus*, *Annona squamosa* Linn., crude plant extract, acaricide, biocontrol

Introduction

Rhipicephalus microplus (Acari: Ixodidae), known as cattle tick, is considered as one of the blood-sucking arthropods/ ectoparasites that are serious threats to a variety of domestic and wild animals throughout the world. Infestation by *R. microplus* may result in severe losses of dairy and meat productions of cattle and goats, especially in the tropical and subtropical regions. In addition, *R. microplus* is also identified as an important vector that transmitting several pathogens to humans and animals, including *Babesia bovis*, *Babesia bigemina* and *Anaplasma marginale*[1]. The chemical acaricides have often been used for controlling infestation by *R. microplus* ticks. However, there are a number of undesirable consequences. Since acaricidal chemicals have non-specific harmful actions to both living creatures and environments, resulting in environmental pollution, contamination in milk and meat products, tick's resistance, and subsequent increases in cost for controlling measures and productions[2]. Therefore, several countries are developing sustainable, alternative methods for tick control. One is the use of extracts from herbal plants as they have been found to have many advantages,

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including effective acaricidal activities, precise actions against various species of ticks, and also biodegradable properties, which are unlikely to contaminate and damage the environments, humans and animals[3].

Annona squamosa Linn. (Custard apple) belongs to the Annonaceae family. This plant is native to the West Indies and can be commonly cultivated throughout India and many Asian countries. *A. squamosa* Linn. is deciduous trees with small well-branches and shrubs where bearing edible fruits and has been found to possess a variety of medicinal properties, such as insecticidal, free radical scavenging, hypoglycemic and anti-diabetic activities[4]. Moreover, several bioactive compounds have been isolated from *A. squamosa* Linn. leaves, barks and fruits, such as β-caryophyllene, α-pinene, α-humulene, α-gurjunene and limonene, which were employed as pesticidal and parasiticidal agents[5,6]. However, to the best of our knowledge, there is limited information of using the extracts derived from *A. squamosa* Linn. leaves as biological control agents against *R. microplus* ticks.

Objective

The aim of this study was to determine the effective concentrations and storage durations of crude hexane extract of *A. squamosa* Linn. leaves for controlling against the engorged female *R. microplus* ticks.

Materials and Methods

Preparation of *A. squamosa* Linn. extract

A. squamosa Linn. leaves were collected from Pakse district, Champasak province, in the Southern region of Laos PDR. *A. squamosa* Linn. leaves were dried at 40°C for 72 h, ground into fine powder and sieved through the mesh with 5-mm pore's size. Hexane was added to the ground material at the ratio of 30% (w/v) and shaken at 200 rpm for 72 h. Subsequently, the mixture was filtered through Whatman filter paper No. 1 and evaporated at 45°C by vacuum rotary evaporator. After that, the concentrated crude extract was collected, transferred to glass vial and kept at 4ºC for long term storage[5]. The crude extract was re-suspended in 2% (w/v) tween solution (diluted with sterile distilled water) at the appropriate concentrations (4,000-32,000 ppm) before being used for further experiment.

Collection and preparation of engorged female *R. microplus* ticks

The engorged female *R. microplus* ticks were collected in the morning from naturally infested cattle pasture on a local ranch in Laos PDR. The cattle were free from any acaricidal treatments for at least 45 days prior the collection process. The appropriate sites of the cattle for collecting engorged female ticks were perineal area, external ear, udder, scrotum and sternum. Next, the engorged female ticks were washed with sterile water, dried using paper towels, and placed in plastic boxes with the perforated cover to allow ventilation. The body weight of each female adult ticks ranged between 0.1 - 0.25 with the average weight of 0.118 g. These female ticks were kept under laboratory conditions at 27±1.5°C and 70 – 80% relative humidity (RH)[7, 8].

Evaluation of acaricidal and egg laying inhibition activities of *A. squamosa* Linn. extract

The experiment was performed in four replicates using ten female ticks for each replicate (n=10). Female ticks were individually immersed into different concentrations of crude hexane *A. squamosa* Linn. extract, i.e., 4,000, 8,000, 16,000 to 32,000 ppm, for 2 minutes. Female ticks in the control group were treated with 2% tween solution (diluted in sterile distilled water). Treated ticks were then removed and placed separately in each glass vials layered with moist filter paper. The vials were placed in the incubator at 27±1.5°C and 70–80% RH. These female ticks were monitored for their mortality for consecutive four days. The survival female ticks (n=10) were also measured for total weight of their laid eggs. Index of egg laying (IE) and % inhibition of egg laying (%IE) were calculated by formulas below[9]

$$IE = \frac{\text{Weight of laid eggs (g)}}{\text{Weight of females (g)}}$$

$$\%IE = \frac{\text{IE control group - IE treated group}}{\text{IE control}} \times 100$$

Effect of time storage on the efficacy of *A. squamosa* Linn. extract

The crude hexane extract of *A. squamosa* Linn. leaves was stored at 4°C in the dark for 1 or 90 days. After that, efficacy of the extract at 16,000 ppm was evaluated on engorged female ticks as described in previous section.

Statistical analysis

The average percentages of mortality and inhibition of egg laying of adult female ticks were analyzed by One-way ANOVA. The experimental treatments were compared using Duncan's multiple range test. The significant difference between treatments was determined at 95% confidence (P<0.05). The lethal concentration 99% (LC_{99}) was calculated by Probit analysis.

Results

In this study, the different concentrations of *A. squamosa* Linn. leaf extract were evaluated for their acaricidal properties against engorged, adult female *R. microplus* ticks. The results showed that leaf extract of *A. squamosa* Linn. at 16,000 and 32,000 ppm concentrations induced significantly high levels of tick mortality (Table 1), which both resulting in killing 95.00±5.00% of ticks within 48 h and 100% within 72 h (P<0.05). The action of *A. squamosa* Linn. extract against female ticks was clearly in a dose-dependent manner with calculated LC_{99} value at 11,157 ppm. No dead ticks were found in the control group (treated with 2% tween solution). In addition, more than 50% of tick mortality was observed as early as 12 and 24 h when applying with *A. squamosa* Linn. leaf extract at the concentrations of 32,000 and 16,000 ppm, respectively.

Table 1 Mortality of *R. microplus* after treatments with various concentrations of *A. squamosa* Linn. leaf hexane extract.

Concentration of *Annona squamosa* Linn. leaf extract	%mortality					
	3h	6h	12h	24h	48h	72h
4,000	0.00±0.00^{b}	0.00±0.00^{b}	5.00±5.00^{bc}	5.00±5.00^{b}	25.00±5.00^{c}	25.00±5.00^{c}
8,000	0.00±0.00^{b}	0.00±0.00^{b}	10.00±5.77^{bc}	30.00±17.32^{ab}	65.00±12.58^{b}	65.00±12.58^{b}
16,000	0.00±0.00^{b}	10.00±5.77^{b}	25.00±5.00^{b}	55.00±15.00^{a}	95.00±5.00^{a}	100.00±0.00^{a}
32,000	15.00±9.57^{a}	30.00±5.77^{a}	55.00±12.58^{a}	65.00±12.58^{a}	95.00±5.00^{a}	100.00±0.00^{a}
Control (2%tween)	0.00±0.00^{b}	0.00±0.00^{b}	0.00±0.00^{c}	0.00±0.00^{b}	0.00±0.00^{d}	0.00±0.00^{d}
P-value	0.0509	0.0001	0.0003	0.0045	0.0001	0.0001

Note: Different letters in the same column indicate significant differences between the treatments (P<0.05).

Percentages of inhibition of *R. microplus* egg laying (%IE) after exposure to various concentrations of *A. squamosa* Linn. leaf hexane extract were shown in Table 2. The results indicated that *A. squamosa* Linn. leaf extract at the concentration of 8000 ppm could significantly and severely inhibit female oviposition when compared to the control group (P<0.05). The maximum %IE was observed when applying with the *A. squamosa* Linn. leaf extract at the concentration of 32,000 ppm (100%), followed by 16,000 ppm (99.71±0.28%), 8,000 ppm (95.93±2.48%) and 4,000 ppm (84.57±6.53%). Although the extract at the concentration of 4,000 ppm was shown to induce significantly lower %IE than those of the higher concentrations (P<0.05), it still found to be able to induce more than 80% IE.

The hexane extract of *A. squamosa* Linn. at 16,000 ppm was clearly shown to be effective against engorged female ticks (Tables 1 and 2). Therefore, this concentration was employed for further evaluation of the effect of storage duration on the efficacy of *A. squamosa* Linn. leaf extract. The results indicated that long term storage of *A. squamosa* Linn. leaf extract significantly

affected its efficacy to induce tick's mortality (P<0.005) (Table 3). However, the extract that was kept for 90 days was still capable of killing as high as 75.00±9.57% and 80.00±8.16% of ticks within 48 and 72 h, respectively.

The results of inhibition of *R. microplus* egg laying (%IE) after being exposed to 16,000 ppm of *A. squamosa* Linn. extract that was stored for 1 or 90 days were shown in Table 4. The results showed that long term storage of the extract at 4 °C did not affect %IE of engorged female ticks. Also, the efficacy of the extract was still as high as 95.53±2.92% after 90 days of storage.

Table 2 Percentages of inhibition of *R. microplus* egg laying (%IE) after treatments with various concentrations of *A. squamosa* Linn. leaf extract.

Concentration of *Annona squamosa* Linn. leaf extract	Weight of female ticks (g)	Weight of egg mass of female ticks (g)	Index of egg laying (IE)	Inhibition of egg laying (%IE)
4,000	0.113±0.01	0.0124±0.01^{b}	0.1034±0.04^{b}	84.57±6.53^{b}
8,000	0.120±0.02	0.0034±0.01^{c}	0.0272±0.02^{c}	95.93±2.48^{a}
16,000	0.117±0.01	0.0002±0.01^{c}	0.0019±0.01^{c}	99.71±0.28^{a}
32,000	0.122±0.01	0.0000±0.00^{c}	0.0000±0.00^{c}	100.00±0.00^{a}
Control (2%tween)	0.115±0.01	0.0750±0.01^{a}	0.6533±0.03^{a}	0.00±0.00^{c}
P-value	0.9365	0.0001	0.0001	0.0285

Note: Different letters in the same column indicate significant differences between the treatments (P<0.05).

Table 3 Mortality of *R. microplus* after treatments with 16,000 ppm of *A. squamosa* Linn. leaf extract that was stored for 1 or 90 days.

Treatment-storage duration	%mortality					
	3h	6h	12h	24h	48h	72h
Annona squamosa-1 day	0.00±0.00	6.25±6.25^{a}	6.25±6.25^{ab}	25.00±0.00^{ab}	100.00±0.00^{a}	100.00±0.00^{a}
Annona squamosa-90 days	0.00±0.00	0.00±0.00^{b}	20.00±8.16^{a}	40.00±14.14^{a}	75.00±9.57^{b}	80.00±8.16^{b}
Control (2%tween)	0.00±0.00	0.00±0.00^{b}	0.00±0.00^{b}	0.00±0.00^{b}	0.00±0.00^{c}	0.00±0.00^{c}
P-value	0.0000	0.4055	0.0102	0.0209	0.0001	0.0001

Note: Different letters in the same column indicate significant differences between the treatments (P<0.05).

Table 4 Percentages of inhibition of *R. microplus* egg laying (%IE) after treatments with 16,000 ppm of *A. squamosa* Linn. leaf extract that was stored for 1 or 90 days.

Treatment-storage duration	Weight of female ticks (g)	Weight of egg mass of female ticks (g)	Index of egg laying (IE)	Inhibition of egg laying (%IE)
Annona squamosa-1 day	0.108±3.30	0.000±0.00^{b}	0.000±0.00^{b}	100.00±0.00^{a}
Annona squamosa-90 days	0.124±7.37	0.005±0.01^{b}	0.046±0.03^{b}	95.53±2.92^{a}
Control (2%tween)	0.122±5.60	0.109±0.01^{a}	1.031±0.10^{a}	0.00±0.00^{b}
P-value	0.196	0.0001	0.0004	0.1768

Note: Different letters in the same column indicate significant differences between the treatments (P<0.05).

Discussion and Conclusion

The use of natural bioactive compounds to replace chemical pesticides has played an important role in the recent years. Obviously, this is because the adverse consequences of chemical pesticides, especially in the cattle meat and dairy production industries. This study hence focused on using *A. squamosa* Linn. leaf extract against engorged female *R. microplus* ticks. *A. squamosa* Linn. is widely cultivated in Lao PDR and its leaves are rather considered as agricultural waste without any further values. Therefore, application of this agricultural waste may provide great benefits and opportunities to the farmers to use it as cheap, natural and safe agent for controlling *R. microplus* ticks[10].

The previous report revealed that *A. squamosa* Linn. had a variety of bioactive constitutions, including 4-(2-nitroethyl)-1-((6-O-ß-D-xylopyranosyl-ß Dglucopyranosyl) -oxy) benzene, anonaine, benzyltetrahydroisoquinoline, borneol, camphene, camphor, car-3-ene, carvone, ß-caryphyllene, eugenol, farnesol, geraniol, 16-hentriacontanone, hexacontanol, higenamine, isocorydine, limonine, linalool, linalool acetate, menthone, methylanthranilate, methylsalicylate, methylheptenone, p-(hydroxybenzyl)-6, 7-(2-hydroxy,4-hydro) isoquinoline, n-octacosanol, á-pinene, ß-pinene, rutin, stigmasterol, ß-sitosterol, thymol and n-triacontanol[11]. Moreover, alkaloid extracts from *A. squamosa* Linn. at the concentrations of 50 - 200 ppm were shown to have larvicidal, chemosterilant and growth-regulating activities against *Anopheles stephensi*[5]. Also, sesquiterpenes and monoterpenes, which were considered as major bioactive compounds of *Annona* species, have been applied as insecticidal agents against the cabbage looper (*Trichoplusia ni*)[12]. Moreover, the acaricidal efficacy of *A. squamosa* Linn. seed extracts was previously evaluated against *R. microplus*, and the studies showed that its seed extract at the concentration of 8% could kill up to 70.8% of ticks after 24 h of application and it also severely suppressed the egg production of engorged female ticks[13]. In addition, the crude ethanol extract of *A. squamosa* Linn. seeds at 10% concentration could kill 87% of *R. microplus* within 48 h by immersion technique[14]. Furthermore, the adulticidal and larvicidal activities of *A. squamosa* Linn. leaf extract against *Haemaphysalis bispinosa* were also assessed and the results showed that application of crude hexane extract of *A. squamosa* Linn. leaves at 2,500 ppm resulted in as high as 100% mortality of *H. bispinosa* adults within 24 h and the lethal concentration that killed 50% of *H. bispinosa* adults (LC_{50}) was determined at 145.39 ppm[5]. The results of this study may suggest that crude hexane extract of *A. squamosa* Linn. leaves was highly effective at killing engorged female *R. microplus* ticks and also severely inhibiting their egg laying. Although long term storage (90 days) could reduce its efficacy to induce tick's mortality, its ability to suppress tick's egg laying still remained stable. The future development of *A. squamosa* Linn. extracts as insecticides should thus consider the methods for preservation of its efficacy for both killing and inhibiting of insect oviposition.

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References

1. Rosado-Aguilar JA, Aguilar-Caballero A, Rodriguez-Vivas RI, Borges-Argaez R, Garcia-Vazquez Z, Mendez-Gonzalez M. Acaricidal activity of extracts from *Petiveria alliacea* (Phytolaccaceae) against the cattle tick, *Rhipicephalus (Boophilus) microplus* (Acari: ixodidae). Veterinary Parasitology 2010; 168(3-4): 299-303.
2. Pirali-Kheirabadi K, Silva JTd. *In Vitro* Assessment of the Acaricidal Properties of *Artemisia annua* and *Zataria multiflora* Essential Oils to Control Cattle Ticks. Iranian Journal of Parasitology 2011;6(1):58-63.
3. Ghosh S, Sharma AK, Kumar S, Tiwari SS, Rastogi S, Srivastava S, Singh M, Kumar R, Paul S, Ray DD, Rawat AK.. *In vitro* and *in vivo* efficacy of *Acorus calamus* extract against *Rhipicephalus* (*Boophilus*) *microplus*. Parasitology Research 2011;108(2):361-370.

4. Thattakudian Sheik Uduman MS, Sundarapandian R, Muthumanikkam A, Kalimuthu G, Parameswari SA, VasanthiSrinivas TR, Karunakaran G. Protective effect of methanolic extract of *Annona squamosa* L. in isoniazid-rifampicin induced hepatotoxicity in rats. Pakistan Journal of Pharmaceutical Sciences. 2011;24(2):129-134.
5. Bagavan, A, Kamaraj C, Elango G, Abduz Zahir A, Abdul Rahuman A. Adulticidal and larvicidal efficacy of some medicinal plant extracts against tick, fluke and mosquitoes. Veterinary Parasitology 2009; 166(3-4): 286-292.
6. Marta A, Arriaga C, Machado M.I.L, Craveiro AA, Dourado RCM. Composition of the root oil of *Annona squamosa* L. from Brazil. Journal of essential oil research 1996.
7. Fernández-Salasa A, Alonso-Díaz MA, Acosta-Rodríguez R, Torres-Acosta JFJ, Sandoval-Castro CA, Rodríguez-Vivas RI. *In vitro* acaricidal effect of tannin-rich plants against the cattle tick *Rhipicephalus* (*Boophilus*) *microplus* (Acari: Ixodidae). Veterinary Parasitology 2011;175: 113–118.
8. Ribeiro VLS, Toigo E, Bordignon SAL, Gonalves K, von Poser G. Acaricidal properties of extracts from the aerial parts of *Hypericum polyanthemum* on the cattle tick *Boophilus microplus*. Veterinary parasitology 2007;147(1-2):199-203.
9. Silva WC, Martins JR, de Souza HE, Heinzen H, Cesio MV, Mato M, Albrecht F, de Azevedo JL, de Barros NM. Toxicity of *Piper aduncum* L. (Piperales: Piperaceae) from the Amazon forest for the cattle tick *Rhipicephalus (Boophilus) microplus* (Acari: Ixodidae). Veterinary Parasitology 2009;164(2-4):267-274.
10. Theppavong B, Khamphan K, Vonghachack S. Conservation and management of forest genetic resources in Lao DPR. Asia Pacific Forest Genetic Resources. 2001; 1(1):29-34.
11. Patel JD, Kumar V. *Annona squamosa* L.: Phytochemical analysis and Antimicrobial Screening. Journal of Pharmacy Research 2008;1(1):34-38.
12. De Cassia Seffrin R, Shikano I, Akhtar Y, Isman MB. Effects of crude seed extracts of *Annona atemoya* and *Annona squamosa* L. against the cabbage looper, *Trichoplusia ni* in the laboratory and greenhouse. Crop Protection. 2010; 29(1):20-24.
13. Magadum S, Mondal D, Ghosh S. Comarative efficacy of *Annona squamosa* and *Azadirachta indica* extracts against *Boophilus microplusIzatnagar* isolate. Parasitology Research. 2009;105(4):1085-10891.
14. Chungsamarnyart N, Jiwajida S, Rattanakreetakul C, Jansawan W. Practical Extraction of Sugar Apple Seeds against Tropical Cattle Ticks. Faculty of Veterinary Medicine, Kasetsart University 1991;25:101-105.