

Acaricidal Activity of Crude Extract of *Annona squamosa* against *Hyalomma anatolicum* (Ixodoidea: Ixodidae)

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Abstract

The acaricidal activities of crude ethanolic extract of the leaves of *Annona squamosa* (annonaceae) was assessed against flat larvae and engorged female adults *Hyalomma anatolicum* using larval (LIT) and adult (AIT) immersion test, respectively. In the LIT, six concentrations of the crude extract (150 mg/ml (15%), 75 mg/ml (7.5%), 37.5 mg/ml (3.75%), 18.75 mg/ml (1.875%), 9.375 mg/ml (0.9375%) and 4.6875 mg/ml (0.46875%) with three replicates for each were used. The same concentrations with 6 replicates were used for AIT. The obtained results indicated that the crude ethanolic extract of *A. squamosa* at all concentrations used, is toxic to *H. anatolicum* larvae and adults. In the LIT mortality rate was observed to vary from 14.983 to 100%, 48 h after treatment. The mortality increased with increase concentration. LC₅₀ and LC₉₉ were 1.366% and 10.170%, respectively. On the other hand the effectiveness of AIT treatment against engorged females was assessed by measuring mortality, inhibition of egg production, hatchability and inhibition of reproduction (growth inhibition). AIT showed 100% mortality at the concentration of 15% and 7.5%, egg laying inhibition of 60.365, 62.282, 81.224, 94.117% and hatching inhibition of 28.4194, 80.69516, 88.89439 and 95.40229%, at the concentration of 0.46875, 0.9375, 1.875 and 3.75%, respectively. Our results showed that crude ethanolic extract of *A. squamosa* is a promising botanical acaricide and growth inhibitor against *H. anatolicum*.

Keywords: *Annona squamosa*; Botanical acaricide; Growth inhibitor; Ethanolic extract; *Hyalomma anatolicum*; Immersion test.

Introduction

Tick species of the genera *Hyalomma*, *Amblyomma*, *Boophilus*, *Rhipicephalus* and *Ornithodoros* are commonly found in the Sudan infesting farm and wide animals. *Hyalomma anatolicum* tick is widely distributed and infesting several host species. It represents the potential vector of *thieleria annulata* [1] which is highly pathogenic to foreign breed cattle. This in addition to its role in transmission of *Babesia equi* [2,3] and the general effect of ticks involving decrease in weight gain and milk production.

Control of ticks and tick-borne diseases and pests of public health in the Sudan, relies mainly on application of synthetic pesticides. The misuse and abuse of these chemicals have created numerous detrimental hazards to human and animal health and affected other non-target organism, and the whole environmental [4] and even contamination of milk and meat product with insecticide residues [5], beside the emergence of resistant strains to these chemicals. Several environmentally sound measure were attempt for pest control as safe alternative to synthetic chemicals, the most important of which are the application of biological control and use of bio pesticides from microbial and plant origin. The natural chemical compounds found in plants, which termed phytochemicals, represent untapped reservoir of biologically active ingredient required for different purpose. These compounds have been perceived by the general public as a relatively safe and pose slight risk to the environment with minimal impact to animal and human health [6]. Botanical pesticides have various effects against ticks such as reducing tick feeding, molting, fecundity and

viability of eggs. Therefore, botanical pesticides have many advantage over synthetic chemicals i.e., minimal mammalian toxicity, impact on pollinators and natural enemies and environmental pollution, beside it being less expensive and easily available [7].

The Sudan flora is rich in plants containing active properties for controlling pests and diseases in the field of agriculture of public health [8,9]. Therefore the aim of this study was to evaluate the acaricidal activity of the crude extract of leaves from *Annona squamosa* against *H. anatolicum* larvae and adults.

Materials and Methods

Plant material and extraction

The leaves of *A. squamosa* (Annonaceae) were collected from Kurdofan State, western Sudan, in September 2013 and the taxonomic identification was performed by botanists in the Medicinal and Aromatic Plants Research Institute (MAPRI), Khartoum, Sudan. The voucher specimen was numbered and deposited in the pesticide test laboratories within the Department of Entomology and Ticks, Veterinary Research Institute (VRI), Khartoum, Sudan. The leaves were shed-dried for 3 days, and ground into small pieces using an electric stainless steel grinder. The powdered leaves (500 g) were extracted with 95% ethanol by soaking (5 days) and re-extracted 2 times. The extract was vacuum-filtered (Whatman No.1) and evaporated at 40°C by vacuum rotary evaporator. The dried extract was transferred to pre-weighed vial. The vial was re-weighed to determine extract weight and yield percentages were calculated as followed: Yield percentage=100(weight of extract/weight of plant

material). The crude extract in the glass vials was kept at 4°C until use [10].

Ticks collection and maintenance

Engorged female ticks were collected from naturally infested cattle in dairy farms at Soba/Khartoum and transferred to the Department of Entomology and Ticks, VRI, Khartoum, Sudan. The ticks were identified according to Hoogstraal [11]. Engorged female *H. anatolicum* ticks were selected, kept in Petri-dishes and maintained in an incubator at 27 ± 1°C and 75-80% relative humidity (RH) to provide optimum conditions for oviposition [12].

The eggs produced were collected daily using a camel-hair brush in labeled (tick species and date of oviposition) glass specimen tubes. A hole of 1.6 cm in diameter was made in the rubber lid of each tube and closed by a film of nylon gauze. The glass containing the eggs, were kept at the same condition to hatch. One -two weeks-old larvae were used for larval immersion test (LIT). The rest of larvae and developing nymphs, and adults were fed on clean, healthy rabbits according to Baily [13]. Engorged females, 24 hours post drop off, were used for adult immersion test (AIT).

Bioassay tests

Larval immersion test (LIT): 12-14 day old unfed larvae of *H. anatolicum* were investigated using the modified larval immersion test [14] to evaluate the acaricidal activity of *A. squamosa* leaves extract. Weight of 15 g of crude extract was dissolved in 100 ml of acetone to prepare 15% stock solution; Tween-20 detergent was used as an emulsifier at a concentration of 0.2%. Then serial dilutions of 75 mg/ml (7.5%), 37.5 mg/ml (3.75%), 18.75 mg/ml (1.875%), 9.375 mg/ml (0.9375%) and 4.6875 mg/ml (0.46875%) were prepared using distilled water. The control solution was set up with acetone and tween-20 in distilled water.

Each concentration and the control were performed in triplicate following the protocol proposed by Shaw [15] with modification [14]. Three ml from each concentration was transferred to Petri dishes (60 mm x 15 mm in diameter), and ~300-500 larvae placed between two whatman No.1 filter papers were immersed for 1 minute.

Approximately each 100 treated larvae were gently picked with a fine brush, transferred and replaced into clean dry labeled filtered paper packet. The open ends of the packets were closed with metallic bulldog clips and incubated at 27 ± 1°C and 75-80% RH for 48 h after which, live and dead larvae were counted to calculate the larval mortality rate corresponding to each concentration used. Larvae that were unable to walk forward, even after stimulated by breathing, were considered dead. The larval percentage mortality rates achieved were corrected according to Abbott's formula [16], recommended by the Food and Agriculture Organization of the United Nation [17]. None of the control replicates showed mortality over 5%. The average corrected mortalities were subjected to a Probit analysis programme [18] for calculating lethal concentrations (LC) to kill 50 and 99% of larvae and their respective 95% confidence interval (CI).

Corrected mortality = $100(\% \text{treated mortality} - \% \text{control mortality}) / 100 - \% \text{control mortality}$.

Adult immersion test (AIT): The AIT [19] was used to determine acaricidal activity of crude ethanolic extract from the leaves of *A. squamosa* against *H. anatolicum* engorged females. Six Groups of 5 engorged females weighting approximately 2 g per each were

immersed for 2 minutes into 10 ml of the respective dilutions, as same as those in the LIT, in a 50 ml Becker flask which was gently agitated. The control group was immersed into Tween-20 and distilled water as recommended by Rosado-Aguilar [20]. Ticks were transferred to double filter paper to remove the excess solution. The dried ticks were placed individually in Petri dish (5.5 cm diameter, 1.5 cm high) and incubated at 27 ± 1°C and 75-80% RH and observed for oviposition. Tick death was confirmed by loss of motility and pedal reflex after exposing to light and also by loss of oviposition property. The eggs in each Petri dish were collected, weighed and the percentage inhibition of egg laying was calculated as follows:

Index of egg laying (IE) = $\text{weight of egg laid (g)} / \text{weight of engorged females (g)}$

%Inhibition of egg laying = $100(\text{control group IE} - \text{treated group IE} / \text{control group IE})$

Lot of eggs from each replicate within the group was placed in labeled glass tubes, incubated at the same conditions for hatching. The number of dead and live larvae and unhatched eggs were determined, and the percentage of hatched eggs was estimated. Estimated reproductive factor (ERF) and the inhibition of reproduction (IR) were calculated [19] as follows:

$$\text{ERF} = 20000XY/Z$$

20000 = average number of eggs per gram

X = weight in gram of eggs produced

Y = estimated percentage hatchability of eggs

Z = weight of experimental female in gram

%Inhibition of reproduction (IR) = $100(\text{Control ERF} - \text{Treated ERF} / \text{Treated ERF})$.

Results

Plant the extraction

Yield percentage of crude ethanolic extract of *A. squamosa* was found to be 17.8%.

Larval Immersion Test (LIT)

The crude ethanolic extract of *A. squamosa* leaves against *H. anatolicum* larvae proved to be toxic; the mortality rates increased with increasing concentration of the extract (Table 1). The probit values of LC50 and LC99 and their respective 95% confidence intervals (CI) were 1.366% (1.1-2.35) and 10.17% (9.32-11.80), respectively. The value of regression coefficient was 0.976 (Table 2).

Adult Immersion Test (AIT)

The results obtained are summarized in Tables 3 and 4. In the present trial the efficacy of the extract of *A. squamosa* against *H. anatolicum* engorged females was assessed by measuring percentage of mortality, inhibition of egg-laying and inhibition of reproduction. Table 3 shows that the high concentration used the high mortality and inhibition of egg laying achieved. The candidate extract induced 100% mortality of the exposed ticks at the highest concentrations used 15% and 7.5% and 60% inhibition of egg laying at the lowest concentration of 0.468%. The percentage hatching inhibition and the estimated

inhibition of reproduction were calculated and found to be coinciding with the concentration strength (Table 4).

Concentration (%)	control	0.46875	0.9375	1.875	3.75	7.5	15
Mortality (%)	2.063	14.983	41.186	44.954	81.569	97.772	100
Corrected mortality (%)	0	13.192	39.95	43.27	81.18	97.72	100

Table 1: Mean mortality and corrected mortality (%) of *Hyalomma anatolicum* larvae exposed to different concentrations of ethanolic extract of the leaves of *Annona squamosa*.

LC50 (CI)	LC99 (CI)	R	Linear equation
1.366(1.1-2.3)	10.17(9.3-11.8)	0.976	Y=1.966+2.672X
LC ₅₀ : Lethal concentration 50% ; CI=confidence interval			
LC ₉₉ :Lethal concentration 99%; R=regression coefficient factor			

Table 2: LC₅₀, CL, LC₉₉, and regression coefficient factor (R) of *Annona squamosa* against *Hyalomma anatolicum*.

Concentration (%)	Tick weight (g)	Mortality (%)	Egg weight (g)	Egg-Laing index	Egg-laying inhibition
Control	0.494	0	0.29067	0.583936	
0.46875	0.313833	0	0.0765	0.231445	60.365
0.9375	0.413	16.7	0.094735	0.220274	62.282
1.875	0.406833	16.7	0.041833	0.109642	81.22
3.75	0.428333	50	0.014983	0.028627	94.117
7.5	0.421833	100			
15	0.411167	100			

Table 3: Mean mortality (%), Egg weight in gram, Egg-laying index, Inhibition of egg laying of *Hyalomma anatolicum* adult females exposed to different concentrations of ethanol extract of the leaves of *Annona squamosa*.

Concentration (%)	Mean mortality(%)	Z	Y	ERF	IR
Control	0	0.494	100	1176789	
0.46875	0	0.313833	71.0580	3464223	70.56
0.9375	16.7	0.413	19.30484	22838.9	98.06
1.875	16.7	0.406833	11.10561	22837.27	98.6
3.75	50	0.428333	4.597701	3216.533	99
7.5	100	0.421833			100
15	100	0.411167			100

Z=Weight of experimental female in gram ; Y=Percent hatchability
ERF=Estimated reproductive factor ; IR=Estimated inhibition of reproduction

Table 4: Mean mortality%, ticks weight, hatchability, estimated reproductive factor and Inhibition of reproduction of *Hyalomma anatolicum* adult females exposed to different concentrations of ethanol extract of the leaves of *Annona squamosa*.

Discussion

The use of natural products mainly the botanical acaricides for the control of ticks has been the focus of research in many countries, principally to withstand the noticeable increasing frequency of acaricides resistant-tick strains. The spread of such tick population shall spoil the imperative efforts of improving livestock and animal intensive industry due to impact of ticks and tick-borne diseases. Acaricides resistance has been documented in the country as a result of continuous uses or abuses of acaricides. This situation has encouraged efforts that should be undertaken to address the emergence of acaricides resistant-ticks of veterinary importance. Hence the present study was conducted to test the ethanolic extract of *Annona squamosa* leaves against *Hyalomma anatolicum* infesting cattle in dairy farms in Khartoum State.

Despite the fact that there are many reports on the acaricidal activity of *A. squamosa* seed extracts yet scanty research has been carried out on toxicity and potency of the leaves extract on ticks. The fruit aqueous extract of *A. squamosa* exhibited larvicidal activity against the cattle tick *Rhipicephalus (Boophilus) microplus* [21]. In the current study, the *A. squamosa* leaves extract at the highest concentrations used induced 100% larval mortality which decreased gradually with concentration declining; the result is similar to that reported by Chungsamarnyart [22] who examined *Rhipicephalus (Boophilus) microplus*. The death of the tested ticks using LIT suggesting that a contact toxic of larvicidal nature is present in the *A. squamosa* leaves ethanolic extract.

This finding is in accordance with results recorded using ethanolic crude extracts of *Ambrosia maritime*, *Guiera senegalensis* [23,24] against larvae of *Hyalomma anatolicum* and Neem Azal extracts against larvae of *H. anatolicum excavatum* [25]. Various plant extracts such as Chamomile flower [26]; *Calea serrata* [27]; *Petiveria alliacea* [20], were found to be toxic to ticks.

The ethanolic extract of *A. squamosa* at the highest concentrations used in the present work exhibited adulticidal activity against *H. anatolicum*. Variation of mortality rate observed might be depending on the concentration of the wet crude extract that contact and entered the tick's skin. In the search of developing herbal acaricides, eight medicinal plants were screened for their efficacy against ticks. Of these only the extracts prepared from the *A. squamosa* seed showed high level of efficacy against *Boophilus microplus* [21,28] reported that the fruit aqueous extract of *A. squamosa* was efficient against adult ticks of *Haemaphysales bispinosa*.

In this study the extract of *A. squamosa*, inhibited egg hatch and reproduction of the treated *H. anatolicum* engorged females. Also Shivaand [28] reported a significant reduction ($P < 0.05$) in the reproductive index of *Boophilus microplus* treated with *A. squamosa* extract by dipping. Alkaloids isolated from *A. squamosa* have showed larvicidal growth regulating and reduced fecundity and fertility in females *Anopheles stephensis* [29].

Hence the obtained results indicated that the crude ethanolic extract of *A. squamosa* is proved to be an effective botanical acaricidal agent against *H. anaticum* larvae and adults and promising growth inhibitor against *H. anaticum*. Additional studies should be conducted to identify the active compounds and clarify their effects on different tick species and strains.

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