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ORIGINAL RESEARCH

Phytochemical Screening and in-vitro Efficacy of *Calpurnia aurea* Against Two Transovarial Vectors: *Amblyomma variegatum and Rhipicephalus microplus*

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Background: Ticks are the second most common vector of human infectious diseases after mosquitoes. Their transovarial transmission contributes to the maintenance of environmental diseases. This study evaluates the phytochemical screening and in vitro efficacy of Calpurnia aurea against the adult survival and egg hatchability of two transovarial transmission vectors: Amblyomma variegatum and Rhipicephalus microplus.

Methods: Plant material was extracted using maceration techniques, and concentrated solutions of 12.5, 25, 50, 100, 200, and 400 ppm were prepared. Distilled water and diazinon were used as negative and positive controls, respectively. Ten adult ticks were exposed for 10 minutes, and dead ticks were counted after 24 hours of recovery. Twenty 15-day-old eggs were immersed for 10 minutes, and after 15 days of incubation, hatched and unhatched eggs were tallied. Preliminary phytochemical constituents were screened. A one-way analysis of variance and the probit regression model determined mean mortality and hatchability and estimated lethal and inhibitory concentrations, respectively.

Results: The ethanolic and aqueous leaf extracts caused 10±0.0% mortality in adult A. variegatum and R. microplus. The effective dose was LC50 of 27 and 29 ppm and LC50 of 37 and 41 ppm, respectively. At 400 ppm, the leaf ethanolic and aqueous extracts showed 18.7±0.9% and 18.3±1.7%; 18.3±1.2% and 19.7±0.3% egg hatching inhibition, respectively. The effective dose had an IC50 of 50 ppm and IC50s of 91 and 79 ppm, respectively. Flavonoids and saponins were found in both leaf and pod extracts.

Conclusion: C. aurea extracts showed a more promising effect on tick survival and hatchability than synthetic diazinon. The susceptibility test indicated that the leaf extract could control vectors and contribute to environmental disease maintenance. Complex phytochemicals, especially phenolic compounds, are additional evidence of effectiveness in vector control. Further investigation of in vivo efficacy and advanced fractionation of phytochemicals is needed.

Keywords: hatchability, mortality, transovarial, phytochemical, vector control

Background

Ticks are the second most common vector of human infectious diseases after mosquitoes. They pose significant public health issues by transmitting several pathogens horizontally and vertically.[1,2] Reports indicate that around 80% of the world's 1.2 billion cattle are affected by ticks and tick-borne diseases (TTBDs), leading to annual losses of $7 billion.[3] Bloodsucking by ticks causes severe economic losses in animal production, resulting in substantial physical damage to livestock, reduced milk production, and lower meat quality.[4] In Ethiopia and other developing countries, animal disease remains one of the principal causes of poor livestock production.[5,6]

Transovarial transmission (TOT) promotes species diversity by allowing diseases to shift hosts between vertebrate species. It is responsible for the spread of diseases from parent to offspring,[7,8] helping maintain the disease in the environment.[9] Engorged ticks lay their eggs in vegetation where many vertebrates live.[10] When a tick bites an infected host, the pathogen enters the tick's stomach lumen, leading to gametogenesis and zygote ookinete production.[9] The

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kinete stage then moves from the midgut into the hemolymph and invades the female tick's tissues, including the ovaries.[11] This process results in a higher density of disease in the vegetation area. Büscher et al[12] demonstrated the intensity of *Babesia ovisi* infection in tick eggs, with prevalence reaching over 90% on the day of tick oviposition.

Several studies have identified the potential for TOT in *Amblyomma variegatum* and *Rhipicephalus microplus*,[11,13,14] which are the primary vectors for bacterial Rickettsiae and protozoan Babesia, respectively.[11,14,15] These parasites rely on transovarial passage to reproduce.[13,16] Rickettsiae are known human pathogens responsible for spotted fever groups, while Babesia affects cattle, dogs, and humans.[16] Interrupting the infection in an eco-friendly way will encourage the goal of integrated vector management (IVM). Using medicinal plants and identifying bioactive chemicals for vector control are significant alternative techniques for IVM.[17]

The use of plants to combat tick vectors is becoming an important area of research. Some examples include extracts from cumin seeds (*Cuminum cyminum), Phyllanthus emblica*, and Tephrosia vogelii.[18,19] The plant *Calpurnia aurea*, a member of the Papilionoideae subfamily, has been traditionally used for tick control, snake bites, and addressing parasitic infestation by local people in different parts of Ethiopia.[17,20,21] *C. aurea* is a small, yellow-flowered shrub that is multi-stemmed and 3–4 m tall.[22,23] Research indicates that *C. aurea* has antibacterial, antioxidant, and killing capacities against lice, maggots, and ticks.[4,24,25] However, limited studies on its impact on egg hatchability and adult survival, particularly in controlling transovarial transmission from adult to egg, have prompted us to evaluate the preliminary phytochemical properties and in vitro efficacy of *C. aurea* against transovarial vectors *A. variegatum* and *R. microplus.*

Materials and Methods

Plant Material Collection and Processing

Full-grown wild plants were selected from Dara District (6° 41′ 94.39″ N, 38° 31′ 8.198″ E), Sidama Region, Ethiopia. Tariku Berihun (PhD) a botanist at Dilla University confirmed taxonomical identification of the plant using Flora of Ethiopia and Eritrea Vol. 03, Page 102–105.[26] The pressed plant specimens were stored in Dilla University's publicly available herbarium. Test plants were collected and dried in the shade and at ambient temperature on a clean paper magazine for two weeks.[27,28] Subsequently, they were ground using a coffee bean grinding machine and sifted through a 200µm mesh. The powdered samples were stored in a tightly closed plastic envelope. The collection of the plant material and related research complies with relevant institutional, national, and international guidelines and legislation.

Plant Extraction

The maceration technique was utilized in the extraction process. For aqueous extraction, 1 g of plant leaves and pod powder was saturated in 1000 ml of cold distilled water in the flask, shaken for 24 hours on an orbital shaker at 110 rpm, and then directly used as a stock solution of 1000 ppm.[29]

For ethanol extraction, 150 g of leaves and 100 g of pod powder were soaked in 1.5 and 1 liters of 97% ethanol, respectively, in a 1:10 ratio,[30] in an Erlenmeyer flask of 500 ml volume. The solutions were shaken for 24 hours in an orbital shaker at 125 rpm. The solutions were filtered using Whatman filter paper. The filtrates were then evaporated in a rotary evaporator at a temperature below 40 °C.[31] Finally, the extracts were labeled and stored until needed.

Preliminary Phytochemical Screening

The preliminary qualitative phytochemical identification of the crude ethanol extract of *C. aurea* leaves and pods were carried out using standard tests performed according to Kenubih et al[32] and Mulata et al.[33]

Alkaloids

To identify alkaloids, the Mayer's test was performed. Briefly, 0.2 g of extracts were added to each test tube, followed by 3 ml of hexane, vigorously agitated, and filtered. A test tube was filled with 5 milliliters of 2% hydrochloric acid (HCL). After boiling and filtering, a few drops of picric acid were added to the liquid. The production of a yellow precipitate suggests the presence of alkaloids.

Anthocyanin

A 1 g sample of each solvent extract was mixed with 5 ml of HCL and filtered. A 5ml solution of 10% ammonium hydroxide was added to the filtrate and thoroughly shaken. Pink, red, or violet colors in the ammoniac phase were regarded as a sign of anthocyanin.

Flavonoids

1 ml of plant extract was mixed with a few drops of 10% lead acetate solution. A yellow precipitate indicated the presence of flavonoids.

Phenolic Compounds

In a test tube, 200 mg of phthalic anhydride was added to the extract, followed by a few drops of strong sulfuric acid. The solution was gently heated for 2–3 minutes. After cooling, the mixture was poured into a beaker containing diluted sodium hydroxide solution and diluted with an equal amount of water. A yellowish precipitate indicated the presence of phenolic compounds.

Tannins

In a test tube, 0.25 g of each solvent extract was heated in 10 ml of distilled water. After boiling, the mixture was filtered and a few drops of 0.1% ferric chloride were added to the filtrate. The formation of a blue-black or greenish-black precipitate indicated the presence of tannins.

Terpenoids

Two milliliters of chloroform were combined with 0.25 gram of each extract. Then, 3 mL of pure sulfuric acid was carefully applied to create a coating. The reddish-brown coloring of the interface showed the presence of terpenoids.

Saponins

To test for saponins, 0.5 g of each extract was boiled with 5 ml of distilled water and then filtered. The filtrate was shaken vigorously. The formation of stable foam indicated the presence of saponins

Steroids

2 g of extract is diluted in 2 mL of acetic anhydride and 1–2 drops of strong sulfuric acid (H2SO4). The combination begins as pink, but as the reaction develops, it turns blue. Finally, it could seem green. This signaled the existence of steroids.

Tick Collection, and Acclimatization

Tick *A. variegatum* and *R. microplus* were collected from cattle that were brought to a veterinary clinic located at (6°47′ 75.91″ N, 38°34′2.261″ E) and from naturally infested cattle pastured in a local grazing area (6°47′73.83″ N, 38°28′4.311″ E) Dara District, Sidama Region, Ethiopia. The samples were then placed in a plastic box lined with cotton wool and sealed with nylon mesh.[34,35] When submitting acaricides, it was checked to ensure that none had been used in the previous 45 days. The insects were then carried to the Dilla University insectary with care, keeping them away from the hot engine of the car to prevent die-off. Ticks were identified and recorded using a stereomicroscope within a few hours of arrival.[10,35]

Adult ticks were acclimatized by being kept in vials with open tops and fully covered with a piece of nylon mesh to ensure protection, sufficient airflow, and humidity. Males and females were stored separately to prevent inbreeding. All vials containing ticks were kept in a plastic box inside environmental chambers (incubators) at 22 °C ± 1°C and 12 hr:12 hr day and night for one week.[36]

Rearing

Engorged female ticks were washed with distilled water and dried upon arrival. The plastic box that is full of watered-down sand was prepared. Up to five clean, engorged female ticks were placed in a beaker, and the beaker was buried in

the sand until the sand-covered half of the beaker was in the plastic box. Incubated at 27 ± 1°C and 85 ± 10% relative humidity. Under optimal rearing conditions, the engorged female ticks of most species begin to lay eggs within 2–7 days. All eggs were collected in a vial seven days after the commencement of incubation. Each vial containing the first week's egg production was labeled with the date, to make the selection more uniform.[37]

Test Bioassay Preparation

1 gram of dry extract and 1000 ml of dechlorinated water were mixed to prepare a 1000 ppm stock. Then, 80 ml of serial dilutions of 12.5, 25, 50, 100, 200, and 400 ppm were prepared in clean beakers. Distilled water was used for negative control and 0.1% diazinon® (Adamitulu Pesticide Processing S.Co., Zeway, Ethiopia) for positive control.[37]

Adult Immersion Test

The adult immersion tests (AIT) were implemented according to Kenubih and Fouche[32,37] with some modifications for acaricidal activity tests of crude extracts of plant materials. Ten adult ticks were exposed to each dilution in a clean Petri dish for 10 minutes by immersion. Positive and negative controls were prepared in the same manner. The test was set up in three replicates.[38,39] Then they were picked out, washed in tap water, and subsequently transferred to another sterile Petri dish and incubated for 24 hours. with an average humidity of 80 ± 10% at 22°C ± 1°C.[40] Finally, dead and live ticks were counted through careful observation under a stereomicroscope. The ticks were judged dead if there were no signs of movement at all or signs of cuticle darkness.[37]

Egg Immersion Test

Envelopes of filter paper (Whatman No. 1) were prepared, and twenty 15-day-old reared eggs were placed in each envelope. The samples were then immersed in prepared serial dilutions for 10 minutes in distilled water, and diazinon was used for the respective negative and positive controls. Finally, the solution was decanted and evaporated from the envelope. Treated eggs were placed in vials and incubated at 28 ± 1°C and 85 ± 5% for 15 days until larvae hatching was completed.[38,39] Each treatment in the experiments was repeated three times. Finally, hatched larvae and unhatched eggs were identified and counted using a stereomicroscope with a range of magnification from x10 to x40.

Data Analysis

Mortality and hatchability data were analyzed using SPSS software version 20. Mean ± standard error (Mean ± SE) expressed by one-way analysis of variance (ANOVA) with multiple comparison tests (Tukey's test) to determine a significant mean mortality and hatchability difference of the concentration, while estimation of the median lethal concentration, LC50, and inhibition concentration, IC50, was made by the probit regression model. The toxicity level of plant extract was classified as follows: non-toxic (IC and LC50 > 1000 ppm); less toxic (IC and LC50 = 500–1000 ppm); moderately toxic (IC and LC50 = 100–500 ppm); strongly toxic (IC and LC50 < 100 ppm), according to Fouche et al.[37]

Results

Effect of C. aurea Leaves Extract Against Adult Ticks

Following a 24-hour post-exposure period, results indicated that there were significantly ($P < 0.05$) increased mortalities at three higher concentrations (100, 200, and 400 ppm) (Table 1). The positive control significantly enhanced tick mortality, but the negative control (distilled water) had no effect (Table 1).

Effectiveness of C. aurea Pod Extract Against Adult Ticks

Adult *A. variegatum* and *R. microplus* treated with different concentrations of ethanol and aqueous extracts of *C. aurea* pod showed significantly higher mortalities at two concentrations (200 and 400 ppm) ($P<0.05$) as shown in Table 2. Here also, the positive control has induced significantly ($P <0.05$) higher tick mortality than pod extracts. The negative control (distilled water) showed no mortality.

Table 1 Adult Tick Mortalities Due to Exposure to Ethanol and Aqueous Extract of C. aurea Leaf

A.variegatum						*R. microplus*			
Ethanol				Aqueous		Ethanol		Aqueous	
C (ppm)	TE	TD	M±SE	TD	M±SE	TD	M±SE	TD	M±SE
0	30	0	0 ± 0.00^{d}	0	0 ± 0.00^{d}	0	0 ± 0.00^{d}	0	0 ± 0.00^{d}
12.5	30	4	1.3 ± 0.33^{d}	0	0 ± 0.00^{d}	2	0.67 ± 0.33^{d}	0	0 ± 0.00^{d}
25	30	14	4.7 ± 0.67^{c}	10	3.3 ± 0.33^{c}	15	5 ± 0.58^{c}	9	3 ± 0.00^{c}
50	30	23	7.7 ± 0.72^{b}	19	6.3 ± 0.88^{b}	21	7 ± 0.58^{b}	17	5.7 ± 0.33^{b}
100	30	30	10 ± 0.0^{a}	29	9.7 ± 0.33^{a}	30	10 ± 0.0^{a}	28	9.3 ± 0.33^{a}
200	30	30	10 ± 0.0^{a}	30	10 ± 0.0^{a}	30	10 ± 0.0^{a}	30	10 ± 0.0^{a}
400	30	30	10 ± 0.0^{a}	30	10 ± 0.0^{a}	30	10 ± 0.0^{a}	30	10 ± 0.0^{a}
+ C	30	30	10 ± 0.0^{a}	30	10 ± 0.0^{a}	30	10 ± 0.0^{a}	30	10 ± 0.0^{a}

Notes: The letter indicators denote significant differences. Identical letters indicate similar effects of the concentration, whereas different letters signify different effects. Mean values with differing letter indicators within each column differ significantly ($P < 0.05$). There is no significant difference in mean values with identical letter indicators ($P < 0.05$).

Abbreviations: C(ppm), Concentrations in Parts per Million; TE, Total Exposed; TD, Total Death; M, Mean; SE, Standard error of the mean; + C, positive control.

Table 2 Adult Ticks Mortalities Due to Exposure to Ethanol and Aqueous Extracts of C.aurea Pod

A.variegatum						*R. microplus*			
Ethanol				Aqueous		Ethanol		Aqueous	
C (ppm)	TE	TD	M±SE	TD	M±SE	TD	M±SE	TD	M±SE
0	30	0	0 ± 0.0^{d}	0	0 ± 0.0^{d}	0	0 ± 0.0^{e}	0	0 ± 0.0^{e}
12.5	30	0	0 ± 0.0^{d}	0	0 ± 0.0^{d}	0	0 ± 0.0^{e}	0	0 ± 0.0^{e}
25	30	0	0 ± 0.0^{d}	0	0 ± 0.0^{d}	0	0 ± 0.0^{e}	0	0 ± 0.0^{e}
50	30	4	1.3 ± 0.3^{d}	2	0.67 ± 0.3^{d}	3	1 ± 0.6^{de}	3	1 ± 0.6^{de}
100	30	9	3 ± 0.6^{c}	7	2.33 ± 0.3^{cd}	8	2.67 ± 0.3^{cb}	6	2 ± 0.58^{cd}
200	30	15	5 ± 0.6^{b}	15	4.6 ± 0.6^{bc}	14	4.67 ± 0.9^{bc}	10	3.33 ± 0.3^{bc}
400	30	18	6 ± 0.0^{b}	16	5 ± 1.15^{b}	16	5.33 ± 0.7^{b}	14	4.67 ± 0.3^{b}
+ C	30	30	10 ± 0.0^{a}	30	10 ± 0.0^{a}	30	10 ± 0.0^{a}	30	10 ± 0.0^{a}

Notes: The letter indicators denote significant differences. Identical letters indicate similar effects of the concentration, whereas different letters signify different effects. Mean values with differing letter indicators within each column differ significantly ($P < 0.05$). Mean values with two superscripts within each column share significant similarity ($P < 0.05$). There is no significant difference in mean values with identical letter indicators ($P < 0.05$).

Abbreviations: C(ppm), Concentrations in Parts per Million; TE, Total Exposed; TD, Total Death; M, Mean; SE, Standard error of the mean; + C, positive control.

Effective Dose Estimates of Extracts Against Adult Ticks

Pearson's goodness of fit in a table is acceptable since the computed χ^2 is smaller than the tabular value for a given degree of freedom (d.f.) (Table 3). The estimated result has a positive slope, indicating a positive association between mortality and concentration.[36]

Comparative Susceptibility of Adult A. variegatum and R. microplus to Leave Extract

In the comparison, *A. variegatum* has shown higher susceptibility to the aqueous and ethanol leaves. (Figure 1). However, both *A. variegatum* and *R. microplus* showed almost similar susceptibility to the ethanol extract at higher lethal concentrations.

Table 3 Effective Doses of C. aurea in Terms of LC_{50} and LC_{90} Against Adult A. variegatum and R. microplus Ticks

A. variegatum					*R. microplus*			
Extract	**LC50 With CL**	**LC90 With CL**	**$\chi 2$ (*df*=4)**	**Slope**	**LC50 With CL**	**LC90 With CL**	**$\chi 2$ (df=4)**	**Slope**
Ethanol leave	27 (22–32)	63 (49–92)	1.53	3.490	29 (24–35)	66 (52–94)	4.19	3.648
Aqueous leave	37 (31–44)	77 (61–107)	2.91	4.063	41 (33–49)	88 (70–125)	2.93	3.800
Ethanol Pod	230 (172–347)	1098 (617 −3038)	3.06	1.891	274 (199–443)	1372 (737–4460)	3.15	1.831
Aqueous Pod	271 (201–420)	1205 (682–3513)	3.60	1.979	386 (258–788)	2289 (1026–12,462)	1.87	1.657

Abbreviations: LC, Lethal concentration; CL, Confidence limit; $\chi 2$, Chi-square; df, degree of freedom.

Effect of C. aurea Leaf Extract Against Tick Eggs

During the post-exposure period, results showed that at two higher concentrations (200 and 400 ppm) of the aqueous and ethanolic *C. aurea* leaf extracts, they significantly ($P< 0.05$) increased the egg-hatching inhibition of *A. variegatum* and *R. microplus*. The diazinon has caused significantly ($P< 0.05$) higher egg hatching inhibition, while the distilled water has shown no hatching inhibition (Table 4).

Effect of C. aurea Pod Extract Against Tick Eggs

Exposure of *A. variegatum* and *R. microplus* to the aqueous and ethanolic *C. aurea* pod extracts significantly ($P <0.05$) increased egg-hatching inhibition at higher concentrations (400 ppm) than the remaining concentration. Diazinon has

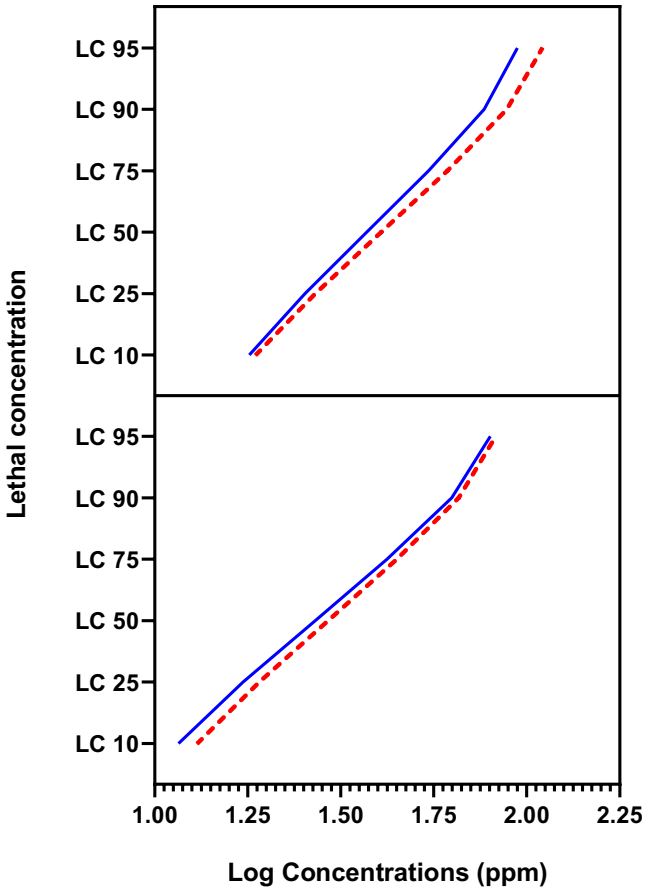

Figure 1 Toxicity and comparative susceptibility of adult *A. variegatum* and *R. microplus* to different concentrations of crude ethanol and aqueous leaf extract. The upper and lower pictured graph represent aqueous and ethanol extracts respectively while the blue and scattered red lines liaised to *A. variegatum* and *R. microplus* respectively.

Table 4 Hatching Inhibition of Ethanol and Aqueous Leave Extract

A.variegatum						*R. microplus*			
Ethanol				**Aqueous**		**Ethanol**		**Aqueous**	
C (ppm)	**TE**	**TUE**	**M±SE**	**TUE**	**M±SE**	**TUE**	**M±SE**	**TUE**	**M±SE**
0	60	0	$0±0.0^{d}$	0	$0±0.0^{d}$	0	$0±0.0^{d}$	0	$0±0.0^{d}$
12.5	60	5	$1.7±0.9^{d}$	1	$0.3±0.3^{d}$	3	$1±1^{d}$	0	$0±0.0^{d}$
25	60	11	$3.7±0.9^{cd}$	3	$1±0.6^{d}$	20	$6.6±0.88^{cd}$	3	$1±1^{cd}$
50	60	35	$10±2.08^{bc}$	15	$5±0.6^{c}$	35	$11.7±1.5^{bc}$	13	$4.3±0.3^{c}$
100	60	48	$16±2.3^{ab}$	35	$11.7±0.3^{b}$	42	$14±2.08^{ab}$	45	$15±0.6^{b}$
200	60	54	$18±0.8^{a}$	50	$16.7±1.9^{a}$	53	$17.7±1.2^{ab}$	52	$17.3±1.5^{ab}$
400	60	56	$18.7±0.9^{a}$	55	$18.3±1.2^{a}$	55	$18.3±1.7^{a}$	59	$19.7±0.3^{a}$
+ C	60	46	$15.3±0.7^{ab}$	43	$14.3±0.7^{ab}$	44	$14.7±0.7^{ab}$	43	$14.3±0.3^{b}$

Notes: The letter indicators denote significant differences. Identical letters indicate similar effects of the concentration, whereas different letters signify different effects. Mean values with differing letter indicators within each column differ significantly ($P < 0.05$). Mean values with two superscripts within each column share significant similarity ($P < 0.05$). There is no significant difference in mean values with identical letter indicators ($P < 0.05$).
Abbreviations: C(ppm), Concentrations in Parts per Million; TE, Total Exposed; TUE, Total unhatched egg; M, Mean; SE, Standard error of the mean; +C, positive control.

Table 5 Hatching Inhibition of Ethanol and Aqueous Pod Extract

A.variegatum						*R. microplus*			
Ethanol				**Aqueous**		**Ethanol**		**Aqueous**	
C (ppm)	**TE**	**TUE**	**M±SE**	**TUE**	**M±SE**	**TUE**	**M±SE**	**TUE**	**M±SE**
0	60	0	$0±0.0^{f}$	0	$0±0.0^{f}$	0	$0±0.0^{e}$	0	$0±0.0^{f}$
12.5	60	0	$0±0.0^{f}$	0	$0±0.0^{f}$	0	$0±0.0^{e}$	0	$0±0.0^{f}$
25	60	2	$0.7±0.7^{ef}$	5	$1.7± 0.9^{ef}$	2	$0.7±0.7^{e}$	4	$1.3±0.7^{ef}$
50	60	12	$4.3±0.7^{de}$	13	$4.3±0.3^{de}$	11	$4±0.6^{d}$	12	$4±0.6^{de}$
100	60	25	$8.3±1.2^{cd}$	25	$7.7±0.7^{cd}$	18	$6.3±0.3^{cd}$	21	$7±0.6^{cd}$
200	60	36	$11.7±0.3^{bc}$	31	$10.3±0.3^{bc}$	20	$7±0.6^{c}$	30	$10±0.6^{bc}$
400	60	40	$13.3±1.7^{ab}$	38	$12.7±1.5^{ab}$	33	$11±0.6^{b}$	34	$11.3±1.2^{b}$
+ C	60	49	$16.3±0.3^{a}$	48	$16±1.2^{a}$	44	$14.7±0.7^{a}$	45	$15±0.0^{a}$

Notes: The letter indicators denote significant differences. Identical letters indicate similar effects of the concentration, whereas different letters signify different effects. Mean values with differing letter indicators within each column differ significantly ($P < 0.05$). Mean values with two superscripts within each column share significant similarity ($P < 0.05$). There is no significant difference in mean values with identical letter indicators ($P < 0.05$).
Abbreviations: C(ppm), Concentrations in Parts per Million; TE, Total Exposed; TUE, Total unhatched egg; M, Mean; SE, Standard error of the mean;+C, positive control.

caused significantly higher egg-hatching inhibition than pod extracts ($P <0.05$). Distilled water showed no hatching inhibition (Table 5).

Effective Dose Estimates of Extracts Against Tick Eggs

Pearson's goodness of fit is acceptable because the calculated $\chi 2$ is less than the tabular value for a given degree of freedom (d.f.) (Table 6). The slope of the calculated value implies a positive correlation between hatchability and concentration.[41]

Comparative Susceptibility of Egg A. variegatum and R. microplus to Leave Extract

In the comparative susceptibility, *R. microplus* has shown higher susceptibility to the aqueous leave plant extracts while in higher concentration *A. variegatum* has shown higher susceptibility to the ethanol leave extract (Figure 2).

Table 6 Effective Dose Analysis of Eggs

A. variegatum					*R. microplus*			
Extract	**IC50 with CL**	**IC90 with CL**	**χ2 (df=4)**	**Slope**	**IC50 with CL**	**IC90 with CL**	**χ2 (df=4)**	**Slope**
Ethanol leave	50 (42–60)	200 (157–274)	8.41	2.143	50 (41–62)	244 (185–353)	7.84	1.874
Aqueous leave	91 (78–107)	293 (233–391)	2.60	2.543	79 (68–89)	193 (162–244)	5.81	3.281
Ethanol pod	169 (138–213)	850 (579–1485)	7.19	1.826	309 (229–473)	2343 (1238–6632)	5.97	1.457
Aqueous pod	188 (149–252)	1268 (775–2670)	5.30	1.547	229 (176–323)	1666 (957–3957)	5.09	1.486

Abbreviations: IC, Inhibition concentration; CL, Confidence limits; χ2, Chi-square; df, degree of freedom.

Phytochemical Screening

The ethanol leaves' and pods' phytochemical constituent profiles are shown in Table 7. In the phytochemical screening test of *C. aurea* ethanol leaf and pod extract, saponin was present.

Discussion

Acaricide resistance in *A. variegatum* and *R. microplus* is widespread in countries where cattle ticks dominate.[42,43] This resistance develops through genetic changes in the tick population, leading to modifications in the target site, enhanced metabolism, or sequestration of the acaricide. Additionally, a reduced ability of chemicals to penetrate the tick's outer protective layers has been observed.[42] These challenges have prompted a shift towards exploring alternative solutions. Medicinal plants, with their specific targets and biodegradability, offer promising interventions. Many plant essential oils have proven effective as acaricides, biopesticides, repellents, and oviposition inhibitors.[43]

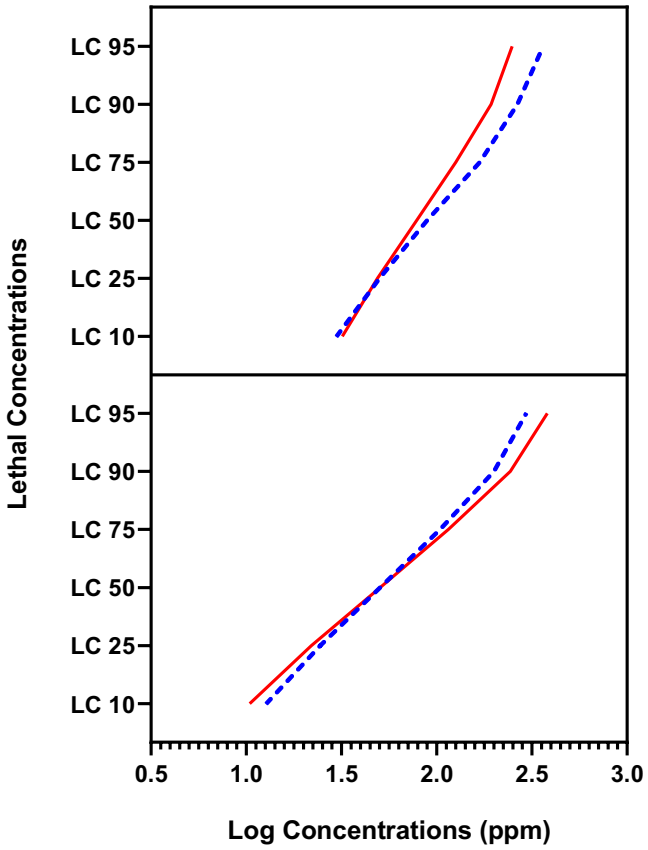

Figure 2 Toxicity and comparative susceptibility of egg *A. variegatum* and *R. microplus* to different concentrations of crude ethanolic and aqueous leaf extract. The upper and lower pictured graph represent aqueous and ethanol extracts respectively while the blue and scattered red lines liaised to *A. variegatum* and *R. microplus* respectively.

Table 7 Phytochemical Screening of the Extracts

Phyto-Constituents	Test	Observation	Plant Extract	
			Leaves	Pod
Alkaloids	Mayer's Test	Yellow	+	–
Anthocyanins	Bontrager Test	No pink color	–	–
Flavonoids	Lead Acetate Test	Yellow	++	+
Phenol	Braemer's Test	Yellowish precipitate	++	–
Tannins	Ferric Chloride Test	Dark Blue-black	++	–
Terpenoids	Salkowski Test	Reddish-brown	+	–
Saponin	Foam Test	Froth	++	++
Steroids	Libermann Burchard Test	No Blue-green color	–	–

Notes: (–) =Absence; (+) = Presence; (++) = Strongly Presence.

Acaricidal Effectiveness of C.aurea Extracts Against Adult Survival and Egg Hatchability

The current study evaluated the phytochemical composition and in vitro efficacy of *Calpurnia aurea* extracts against the adult survival and egg hatchability of two tick species, *Amblyomma variegatum* and *Rhipicephalus microplus*. The results indicated that the ethanolic and aqueous leaf extracts of *C. aurea* significantly reduced the survival of adult ticks and inhibited the hatchability of their eggs. These findings highlight the potential of *C. aurea* as a natural acaricide for tick control.

The LC_{50} values for the ethanolic and aqueous leaf extracts were 27 ppm and 29 ppm for *A. variegatum*, and 37 ppm and 41 ppm for *R. microplus*, respectively (Table 3). These low LC_{50} values suggest that *C. aurea* extracts are highly effective at killing adult ticks at relatively low concentrations. Furthermore, the extracts also significantly inhibited egg hatchability, with the highest concentration (400 ppm) resulting in over 18% inhibition for both tick species. The IC_{50} values for egg hatchability were 50 ppm for *A. variegatum* and 91 ppm and 79 ppm for *R. microplus* with ethanolic and aqueous extracts, respectively (Table 6).

The study demonstrated that the IC_{50} and LC_{50} values of both ethanol and aqueous leaf extracts were less than 100 ppm (Table 3 and Table 6), indicating a strong acaricidal effect as described by Fouche et al.[37] In contrast, the IC_{50} and LC_{50} values for ethanol and aqueous pod extracts were greater than 100 ppm, categorizing them as less toxic. This suggests that acaricidal phytoconstituents are more concentrated in the leaves than in the pods. The LC_{50} of the ethanolic leaf extract of *C. aurea* is lower than that of the aqueous extract against *A. variegatum* and *R. microplus*, indicating higher potency likely due to the polarity of the solvents; ethanol, with both polar and non-polar properties, can extract a wider range of organic compounds compared to water, which is only polar. Notably, the superior potency of the ethanolic leaf extract over the aqueous extract may be due to ethanol's ability to disrupt the wax coating on eggshells, thereby inhibiting egg hatchability.

Compared to synthetic acaricides like diazinon, which are commonly used for tick control, *C. aurea* extracts offer a more sustainable and environmentally friendly alternative. Synthetic acaricides can have negative effects on non-target organisms, contribute to environmental pollution, and lead to the development of resistance in tick populations. In contrast, plant-based acaricides, such as those derived from C. aurea, are biodegradable and less likely to cause resistance due to their complex mixture of bioactive compounds.

When compared with the acaricidal effectiveness of some studied plants such as *C. swynnertonii* in Mkangara,[44] *Laggera oloptera*,[45] *Tagetes patula* in Ismail et al,[46] *T.patula* in Politi et al,[47] and many others, the current study revealed that *C. aurea* leaf extract is more potent than the aforementioned plants. However, it is less effective as compared to other plants such as *Piper tuberculatum* in the study of Braga et al,[48,49] where the LC50 is 5.30 mg/ml. A similar study by Amante and colleagues,[49] reported that the alkaloid solvent of *C. aurea* leaf is more potent, with an LC_{50} of 16.69 ppm.

An important finding of this study is that the two adult tick species, *A. variegatum* and *R. microplus*, exhibited similar levels of susceptibility to leaf extracts, as indicated by the median lethal concentrations (LC_{50} levels) in Figure 1. This suggests that a single application of the extract can effectively control both species when they co-infest the same animal.

Additionally, the study found minimal differences in the susceptibilities of the eggs of both species to ethanol and aqueous leaf extracts, particularly with the ethanol extract, which showed similar LC_{50} levels (Figure 2). This further supports the potential for a single application to manage infestations of both species in the same vegetation.

During the experiment, adult *A. variegatum* and *R. microplus* ticks exhibited shaking and seemed more agitated while trying to sneak out of the solution. It was followed by cuticle darkness, shrinking, and leg paralysis. The cuticle of ticks is made externally by waxes and internally by proteins. Since the more non-polar a chemical compound is, the greater its ability to penetrate through the cuticle, organic extraction may work better in such studies.[49,50] Oliveira et al,[51] also indicated that phenolic compounds are more effective.

Booth et al,[52] described that, during oviposition in ticks, a gene organ applies a superficial wax secretion to the eggs that are synthesized by specialized glandular epithelial cells. The wax prevents egg desiccation, inhibits fungal attack, and causes the eggs to adhere together in a cluster. Proper wax coating is mandatory for the proper hatching of eggs and the maintenance of the fecundity of ticks.[53] In this study, the treated egg tick shows shriveling and characteristically darkening in color, and the eggs are also curled in on themselves, are brittle, and do not adhere together strongly. It is also possible that the active substances found in *C. aurea* might interfere with egg waxes, thereby passing the egg wax coating and killing the embryo.[54]

Phytochemical Constituents of C.aurea Ethanolic Extracts

The phytochemical screening revealed the presence of several bioactive compounds in the leaf and pod extracts of *C. aurea*, including flavonoids, saponins, tannins, and phenolic compounds (Table 7). These compounds, known for their acaricidal properties, likely contribute to the observed effects. Flavonoids, for instance, exhibit anti-inflammatory, antioxidant, and antimicrobial activities that could enhance their effectiveness against ticks. However, advanced screening methods like Gas Chromatography and HPLC are needed for better characterization.

The preliminary phytochemical screening suggests that the leaf extract contains phenolic compounds, which are believed to act on the tick's central nervous system. According to Heong et al,[55] most fast-acting insecticides inhibit nerve impulse transmission and neurotransmitter activity. Specifically, they disrupt the binding of the neurotransmitter mediator GABA (γ-aminobutyric acid chloride flux) to its nerve receptors, as described by Cole et al[56] and Abbas et al.[57] The rapid acaricidal effect of C. aurea against adult ticks observed in this study may be due to its phenolic content.

Tannin and saponin-rich plant extracts have demonstrated acaricidal activity against *R. microplus* larvae.[58] Saponins disrupt cell membranes, leading to cell death, while tannins interfere with protein synthesis and enzyme activity, affecting tick development and survival. Dantas et al,[59] noted the presence of phenolic compounds used as an alternative control against adult *R. microplus*. Terpenoid compounds have also been shown to impact female fertility and egg viability. Alkaloids and phenolic compounds are among the most toxic natural substances and possess neurotoxic properties that cause tick mortality.[60,61] The inhibition of egg hatching by the ethanol leaf extract of *C. aurea* may result from a complex mixture of alkaloids, phenols, tannins, terpenoids, and saponin compounds.

Conclusion

In conclusion, the ethanolic and aqueous leaf extracts of *C. aurea* have shown promising acaricidal properties against *A. variegatum* and *R. microplus*, affecting both adult survival and egg hatchability. The presence of phytochemicals such as flavonoids, saponins, tannins, and phenolic compounds likely contributes to these effects. *C. aurea* extracts provide a potential alternative to synthetic acaricides, offering a more environmentally friendly and sustainable approach to tick control. Future studies should focus on evaluating the in vivo efficacy of these extracts, as well as isolating and identifying the specific bioactive compounds responsible for the acaricidal activity and parasite transmission.

Data Sharing Statement

All data generated or analyzed during this study are included in this manuscript.

Ethics Approval and Consent to Participate

Ethical approval was obtained from the Research Ethics and Review Committee of the CNCS, Department of Biology, Dilla University, Dilla, Ethiopia. No experiments on humans or live animals were involved in this study.

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References

1. Abubakar M, Perera PK, Iqbal A. Introductory chapter : ticks and tick-borne pathogens. in *ticks and tick-borne diseases*. *IntechOpen*. 2018;2018:1–7.
2. Chmelař J, Kotál J, Kopecký J, Pedra JH, Kotsyfakis M. All for one and one for all on the tick – host battle fi eld. *TRENDS Microbiol*. 2016;1–10. doi:10.1016/j.pt.2016.01.004
3. Wellington KW, Leboho T, Sakong BM, et al. Further studies on South African plants: acaricidal activity of organic plant extracts against Rhipicephalus (Boophilus) microplus (Acari: ixodidae). *Vet, Parasitol*. 2017;234:10–12. doi:10.1016/j.vetpar.2016.12.014
4. Gizaw A. *In Vitro Efficacy of Methanolic Extracts of Vernonia Amygdalina, Croton Macrosta Chyus, Ricinus Communis and Petroleum Ether Extract of Millettia Ferruginea Against Bovicola Ovis and Rhipicephalus (Boophilus) Decoloratus*. Addis Ababa University; 2015.
5. Sori T, Bekana M, Adugna G, Kelbessa E. Medicinal plants in the ethnoveterinary practices of borana pastoralists, Southern Ethiopia. *Int J appl Res Vet Med*. 2004;2:220–225.
6. Hurtado OJB, Giraldo-Ríos C. Economic and health impact of the ticks in production animals. in *ticks and tick-borne pathogens*. *IntechOpen*. 2016;1:13. doi:10.5772/57353
7. Bergren NA, Kading RC. The ecological significance and implications of transovarial transmission among the vector-borne bunyaviruses : a review. *Insects*. 2018;9(4):1–21. doi:10.3390/insects9040173
8. Kotál J, Langhansová H, Lieskovská J, et al. Modulation of host immunity by tick saliva Jan. *J Proteomics*. 2016;14:58–68.
9. Ravindran R, Hembram PK, Kumar GS, et al. Transovarial transmission of pathogenic protozoa and rickettsial organisms in ticks. *Parasitol Res*. 2023;1:1–14.
10. Latif AA, Walker AR. An introduction to the biology and control of ticks in Africa. *ICTTD-2 Project*. 2016;2016:1.
11. Obregón D, Corona-González B, Díaz-sánchez AA, Armas Y. Efficient transovarial transmission of babesia spp. in Rhipicephalus microplus ticks fed on water Buffalo (Bubalus bubalis). *Pathogens*. 2020;9(4):1–10. doi:10.3390/pathogens9040280
12. Büscher G, Friedhoff KT, El-Allawy TAA. Quantitative description of the development of Babesia ovis in Rhipicephalus bursa (hemolymph, ovary, eggs). *Parasitol Res*. 1988;74(4):331–339. doi:10.1007/BF00539454
13. Socolovschi C, Mediannikov O, Raoult D, Parola P. The relationship between spotted fever group Rickettsiae and Ixodid ticks. *Vet Res*. 2009;40 (2):1–20. doi:10.1051/vetres/2009017
14. Howell JM, Ueti MW, Palmer GH, Scoles GA, Knowles DP. Transovarial Transmission Efficiency of Babesia bovis Tick Stages Acquired by Rhipicephalus (Boophilus) microplus during Acute Infection. *J Clin Microbiol*. 2007;45(2):426–431. doi:10.1128/JCM.01757-06
15. Socolovschi C, Huynh TP, Davoust B, et al. Transovarial and trans-stadial transmission of Rickettsiae africae in Amblyomma variegatum ticks. *Clin Microbiol Infect*. 2009;15:317–318. doi:10.1111/j.1469-0691.2008.02278.x
16. Socolovschi C, Raoult D. Typhus fevers and other rickettsial diseases, historical. *Vet Res*. 2009;40:100–120.
17. Meresa A, Fekadu N, Degu S, Tadele A, Geleta B. An ethno botanical review on medicinal plants used for the management of hypertension. *Clin Exp Pharmacol*. 2017;07:1–16.
18. Abdisa T. Review on Traditional Medicinal Plant and its Extract Effect on Tick Control in Ethiopia. *J Vet Med Res*. 2017;4:1082.
19. Bagavan A, Kamaraj C, Elango G, Zahir AA, Rahuman AA. Adulticidal and larvicidal efficacy of some medicinal plant extracts against tick, fluke and mosquitoes. *Vet, Parasitol*. 2009;166(3–4):286–292. doi:10.1016/j.vetpar.2009.09.007
20. Giday M, Teklehaymanot T, Animut A, Mekonnen Y. Medicinal plants of the Shinasha, Agew-awi and Amhara peoples in northwest Ethiopia. *J Ethnopharmacol*. 2007;110(3):516–525. doi:10.1016/j.jep.2006.10.011

21. Suleman S, Alemu T. Journal of Herbs, Spices & Medicinal A Survey on Utilization of Ethnomedicinal Plants in Nekemte Town, East Wellega (Oromia), Ethiopia. *J Herbs, Spices Med Plants*. 2012;18(1):34–57. doi:10.1080/10496475.2011.645188
22. Adedapo AA, Jimoh FO, Koduru S, Afolayan AJ, Masika PJ. Antibacterial and antioxidant properties of the methanol extracts of the leaves and stems of Calpurnia aurea. *BMC Complement Altern Med*. 2008;8(1):1–8. doi:10.1186/1472-6882-8-53
23. Zorloni A, Penzhorn BL, Eloff JN. Extracts of Calpurnia aurea leaves from southern Ethiopia attract and immobilise or kill ticks. *Vet, Parasitol*. 2010;168(1–2):160–164. doi:10.1016/j.vetpar.2009.10.026
24. Birhan M, Tessema T, Kenubih A, Yayeh M. In vitro antimicrobial evaluation of aqueus methanol extract from Calpurnia aurea (Fabaceae) Leaves. *Vitr Antimicrob Eval Aqueus Methanol Extr from Calpurnia Aurea Leaves*. *Asian J Med Pharm Res*. 2018;8:33–43.
25. Alemu S. *Study on in vitro Louscidal and Acaricidal Properties of Calpurnia Aurea, Otostegia Integrifolia, Nicotiana Tabaccum and Jatropha Curcas Against Bovicola Ovis and Amblyomma Variegatum*. Addis Ababa University; 2015.
26. Hedberg I, Edwards S. *Flora of Ethiopia: PITTOSPORACEAE to ARALIACEAE*. Vol. 3. Sweden: The National Herbarium, BiologyDepartment, Science Faculty, Addis Ababa University, Ethiopia, and The Department of Systematic Botany, Uppsala University; 1989.
27. World Health Organization (WHO). *WHO Guidelines on Good Herbal Processing Practices (GHPP) for Herbal Medicines. Good Herbal Processing Practices (GHPP) for Herbal Medicines*. World Health Organization (WHO); 2017.
28. Waqas Y, Asif H, Sharif A, Riaz H, Bukhari IA. Traditional medicinal plants used for respiratory disorders in Pakistan : a review of the ethno - medicinal and pharmacological evidence. *Med Aromat Plants*. 2018. doi:10.1186/s13020-018-0204-y
29. Mandefro B, Mereta ST, Tariku Y, Ambelu A. Molluscicidal effect of Achyranthes aspera L. (Amaranthaceae) aqueous extract on adult snails of Biomphalaria pfeifferi and Lymnaea natalensis. *Mand Al Infect Dis Poverty*. 2017;6:1–5.
30. Osman IM, Mohammed AS, Abdalla AB. Acaricidal properties of two extracts from Guiera senegalensis J F. Gmel. (Combretaceae) against Hyalomma anatolicum. *Vet, Parasitol*. 2014;199(3–4):201–205. doi:10.1016/j.vetpar.2013.11.010
31. Oshadie G, Silva D, Abeysundara AT, Minoli M, Aponso W. Extraction methods, qualitative and quantitative techniques for screening of phytochemicals from plants. *Am J Essent Oils Nat Prod*. 2017;2017:(5):29–32.
32. Kenubih A, Belay M, Awol N, Birhan M. Extraction and phytochemical screening of Calpurnia aurea leaves and their effect against malopaagus ovinus. *Asian J Med Pharm Res*. 2019;9:1–10.
33. Mulata HN, Gnanasekaran N, Melaku U, Daniel S. Phytochemical screening and assessment of in vitro antioxidant activities of Calpurnia aurea seeds and leaves. *Int J Pharm Pharm Res*. 2015;2:1–12.
34. Petney TN, Pfäffle MP, Sprong H, Mihalca AD. *Ticks of Europe and North Africa*. Springer International Publishing; 2017; doi:10.1007/978-3-319-63760-0
35. Walker AR, Bouattour A, Camicas JL, et al. *Ticks of Domestic Animals in Africa: A Guide to Identification of Species*. Edinburgh Scotland, U.K: The University of Edinburgh. Bioscience Reports; 2014.
36. Nuss AB, Mathew MG, Gulia-Nuss M. Rearing ixodes scapularis, the black-legged tick: feeding immature stages on mice. *J Vis Exp*. 2017;2017:5–9.
37. Fouche G, Adenubi OT, Leboho T, et al. Acaricidal activity of the aqueous and hydroethanolic extracts of 15 South African plants against Rhipicephalus turanicus and their toxicity on human liver and kidney cells. *Onderstepoort J Vet Res*. 2019;86(1):1–7. doi:10.4102/ojvr.v86i1.1665
38. Rosado-aguilar JA, Aguilar-caballero A, Rodriguez-vivas RI, Borges-argaez R, Garcia-Vazquez Z, Mendez-Gonzalez M. Vet. Parasitol Acaricidal activity of extracts from Petiveria alliacea (Phytolaccaceae) against the cattle tick, Rhipicephalus (Boophilus) microplus (Acari : ixodidae). *Vet, Parasitol*. 2010;168(3–4):299–303. doi:10.1016/j.vetpar.2009.11.022
39. Madzimure J, Nyahangare ET, Hamudikuwanda H, et al. Efficacy of Strychnos spinosa (Lam.) and Solanum incanum L. aqueous fruit extracts against cattle ticks. *Trop Anim Health Prod*. 2013;45(6):1341–1347. doi:10.1007/s11250-013-0367-6
40. Juliet S, Ravindran R, Ramankutty SA, et al. Jatropha curcas (Linn) leaf extract -a possible alternative for population control of Rhipicephalus (Boophilus) annulatus. *Asian Pacific J Trop Dis*. 2012;2(3):225–229. doi:10.1016/S2222-1808(12)60051-6
41. Mandefro B, Mereta ST, Ambelu A. Efficacy of Achyranthes aspera (L) as a molluscicidal bait formulation against fresh water snail biomphalaria pfeifferi. *Evidence-Based Complement Altern Med Vol*. 2018;2018:1–7.
42. Guerrero FD, Léonore L, Ricardo JM. Acaricide resistance mechanisms in Rhipicephalus (Boophilus) microplus. *Rev Bras Parasitol Veterinária*. 2012;2961:1–6.
43. Turkson P, Botchey M. Acaricide resistance in the cattle tick, *Amblyomma variegatum*, in the coastal savanna zone of Ghana. *Ghana J Agric Sci*. 1999;32(2):199–204. doi:10.4314/gjas.v32i2.1902
44. Mkangara M. acaricidal activity of Commiphora swynnertonii (Burtt) Stem Bark Extracts against adult Rhipicephalus appendiculatus Newman and Amblyomma variegatum 68. *Am J Res Commun*. 2014;2:82–92.
45. Coulibaly A, Bationo RK, Koala M, et al. Acaricidal activity of three plants extracts from the central region of Burkina Faso on adult *Rhipicephalus (Boophilus) microplus* cattle ticks. *Int J Biol Chem Sci*. 2020;14(5):1511–1519. doi:10.4314/ijbcs.v14i5.2
46. Ismail MSM, Tag HM, Rizk MA. Acaricidal, ovicidal, and repellent effects of Tagetes patula leaf extract against Tetranychus urticae Koch (Acari: tetranychidae). *J Plant Prot Res*. 2019;59:151–159.
47. Politi FAS, Figueira GM, Araújo AM, et al. Acaricidal activity of ethanolic extract from aerial parts of Tagetes patula L. (Asteraceae) against larvae and engorged adult females of Rhipicephalus sanguineus (Latreille, 1806). *Parasites Vectors*. 2012;5(1):1–11. doi:10.1186/1756-3305-5-295
48. Braga AG, Souza KF, Barbieri FD, et al. Acaricidal activity of extracts from different structures of Piper tuberculatum against larvae and adults of Rhipicephalus microplu. *Acta Amaz*. 2018;48(1):57–62. doi:10.1590/1809-4392201700053
49. Amante M, Hailu Y, Terefe G, Asres K. In-Vitro Louscidal and Acaricidal Activities of Alkaloid of Calpurnia aurea Extracts against Linognathus ovillus And Amblyomma variegatum. *Int J Pharm Sci Res*. 2019;10:431–437.
50. Adenubi OT, Fasina FO, Mcgaw LJ, Eloff JN, Naidoo V. South African Journal of Botany Plant extracts to control ticks of veterinary and medical importance : a review. *South African J Bot J*. 2016;105:178–193.
51. Oliveira PR, de Bechara GH, Denardi SE, Pizano MA, Mathias MIC. Toxicity effect of the acaricide fipronil in semi-engorged females of the tick Rhipicephalus sanguineus (Latreille, 1806) (Acari : ixodidae): preliminary determination of the minimu. *Exp Parasitol*. 2011;127(2):418–422. doi:10.1016/j.exppara.2010.09.009
52. Booth TF, Beadle DJ, Hart RJ. The effects of precocene treatment on egg wax production in gene's organ and egg viability in the cattle tick boophilus microplus (Acarina ixodidae): an ultrastructural study. *Exp Appl Acarol*. 1986;2(2):187–198. doi:10.1007/BF01213761

53. Rees HH. Hormonal control of tick development and reproduction. *Parasitology*. 2004;129(S1):127–143. doi:10.1017/S003118200400530X
54. Ravindran R, Juliet S, Sunil AR, et al. Veterinary Parasitology Eclosion blocking effect of ethanolic extract of Leucas aspera (Lamiaceae) on Rhipicephalus (Boophilus) annulatus. *Vet, Parasitol*. 2011;179(1–3):287–290. doi:10.1016/j.vetpar.2011.02.021
55. Heong KL, Tan KH, Fabellar LT, Lu Z. *Research Methods in Toxicology and Insecticide*. International Rice Research Institute; 2011.
56. Cole LM, Nicholson RA, Casida JE. Action of Phenylpyrazole Insecticides at the GABA-Gated Chloride Channel. *Pestic Biochem Physiol*. 1993;46(1):47–54. doi:10.1006/pest.1993.1035
57. Abbas RZ, Zaman MA, Colwell DD, Gilleard J, Iqbal Z. Acaricide resistance in cattle ticks and approaches to its management : the state of play Vet. Parasitol Acaricide resistance in cattle ticks and approaches to its management : the state of play. *Vet, Parasitol*. 2014;203(1–2):6–20. doi:10.1016/j.vetpar.2014.03.006
58. Fernández-salas A, Alonso-Díaz MA, Acosta-Rodríguez R, et al. Veterinary Parasitology In vitro acaricidal effect of tannin-rich plants against the cattle tick Rhipicephalus (Boophilus) microplus (Acari : ixodidae). *Vet Parasitol*. 2011;175(1–2):113–118. doi:10.1016/j.vetpar.2010.09.016
59. Dantas ACS, Freire DP, Souza GR, et al. Acaricidal activity of leaves of Morus nigra against the cattle tick Rhipicephalus microplus. *Arq Bras Med Vet e Zootec*. 2017;69(3):523–528. doi:10.1590/1678-4162-8994
60. Adedapo AA, Otesile AT, Soetan KO. Assessment of the Anthelmintic Efficacy of an Aqueous Crude Extract of Vernonia amygdalina. *Pharm Biol*. 2007;45(7):564–568. doi:10.1080/13880200701498978
61. Fouche G, Sakong BM, Adenubi OT, et al. Investigation of the acaricidal activity of the acetone and ethanol extracts of 12 South African plants against the adult ticks of *Rhipicephalus turanicus*. *Onderstepoort J Vet Res*. 2017;84(1):1–6. doi:10.4102/ojvr.v84i1.1523