

Phytochemical screening and *in-vitro* efficacy of *Calpurnia aurea* against two transovarial vectors: *Amblyomma variegatum* and *Rhipicephalus microplus*

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Abstract

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.*

Conclusions: *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.*

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 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 spacemen 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 $80 \pm 10\%$ at $22^{\circ}\text{C} \pm 1^{\circ}\text{C}$ [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 $28 \pm 1^{\circ}\text{C}$ and $85 \pm 5\%$ 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 $\pm$ standard error (Mean $\pm$ 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).

Insert 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.

Insert table 2

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].

Insert table 3

Comparative susceptibility of adult *A. variegatum* and *R. (B) 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. (B) microplus* showed almost similar susceptibility to the ethanol extract at higher lethal concentrations.

Insert figure 1

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).

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

Insert table 5

Effective dose estimates of extracts against tick eggs

Pearson's goodness of fit is acceptable because the calculated χ^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].

Insert table 6

Comparative susceptibility of egg *A. variegatum* and *R. (B) microplus* to leave extract.

In the comparative susceptibility, *R. (B) 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).

Insert figure 2

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.

Insert table 7

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].

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 LC₅₀ 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 LC₅₀ 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 IC₅₀ 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 IC₅₀ and LC₅₀ values of both ethanol and aqueous leaf extracts were less than 100 ppm (Tables 3 and 6), indicating a strong acaricidal effect as described by Fouche et al. [37]. In contrast, the IC₅₀ and LC₅₀ 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 LC₅₀ 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 ethanol 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. [45], *Laggera oloptera* [46], *Tagetes patula* in Ismail et al. [47], *T. patula* in Politi et al. [48], 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. [49,50], where the LC50 is 5.30 mg/ml. A similar study by Amante and colleagues [50], reported that the alkaloid solvent of *C. aurea* leaf is more potent, with an LC₅₀ 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 (LC₅₀ levels) in Fig. 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 LC₅₀ levels (Fig. 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 [50,52]. Oliveira et al. [53], also indicated that phenolic compounds are more effective.

Booth et al. [54], 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 [55]. 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 [56].

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.* [57], 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. [58] and Abbas *et al.* [59]. 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 [60]. 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., [61], 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 [62,63]. 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.

Declarations

Availability of data and materials

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

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Authors' contributions

N.N. carry out the experiment, prepared the manuscript, and, was a major contributor to the write-up. N.N., B.M., and, D.A. analyzed and interpreted the data and review the manuscript. B.M. Supervised the experiments. All authors read and approved the final 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.

Consent for publication

Not applicable

Competing interests

The authors declare no competing interests.

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Tables

Tables 1 to 7 are available in the Supplementary Files section

Figures

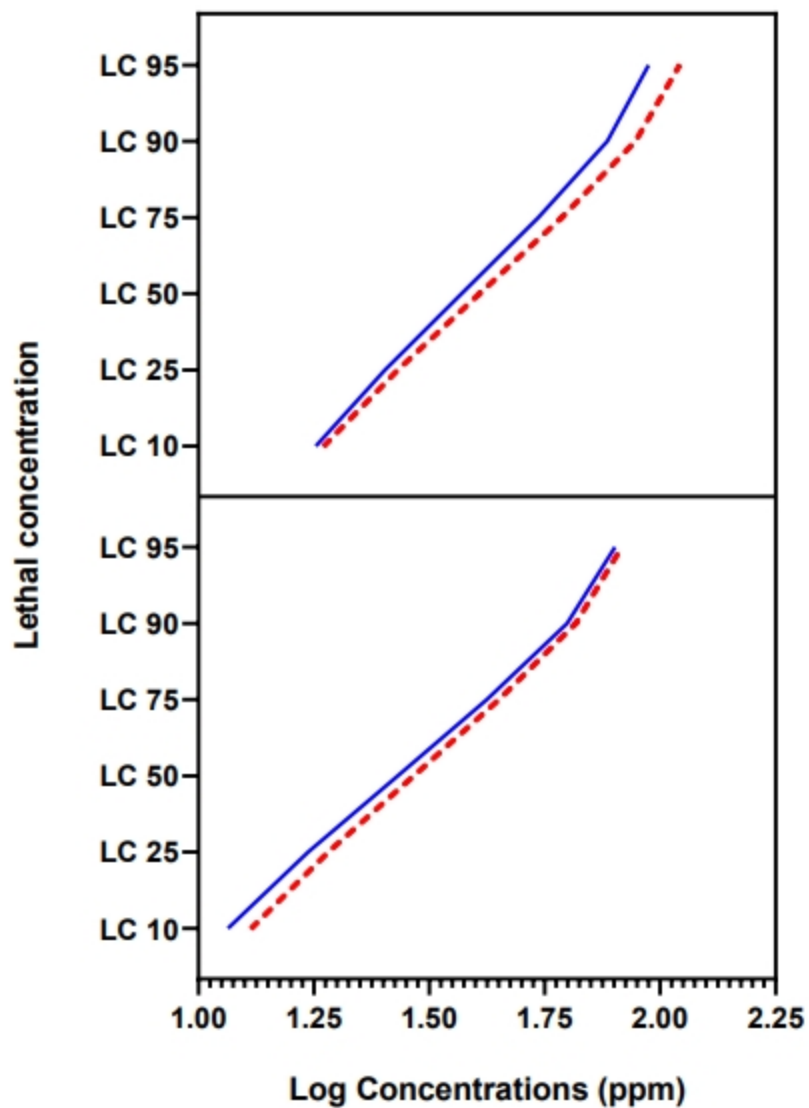


Figure 1

Toxicity and comparative susceptibility of adult *A. variegatum* and *R. (B) 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. (B) microplus* respectively.

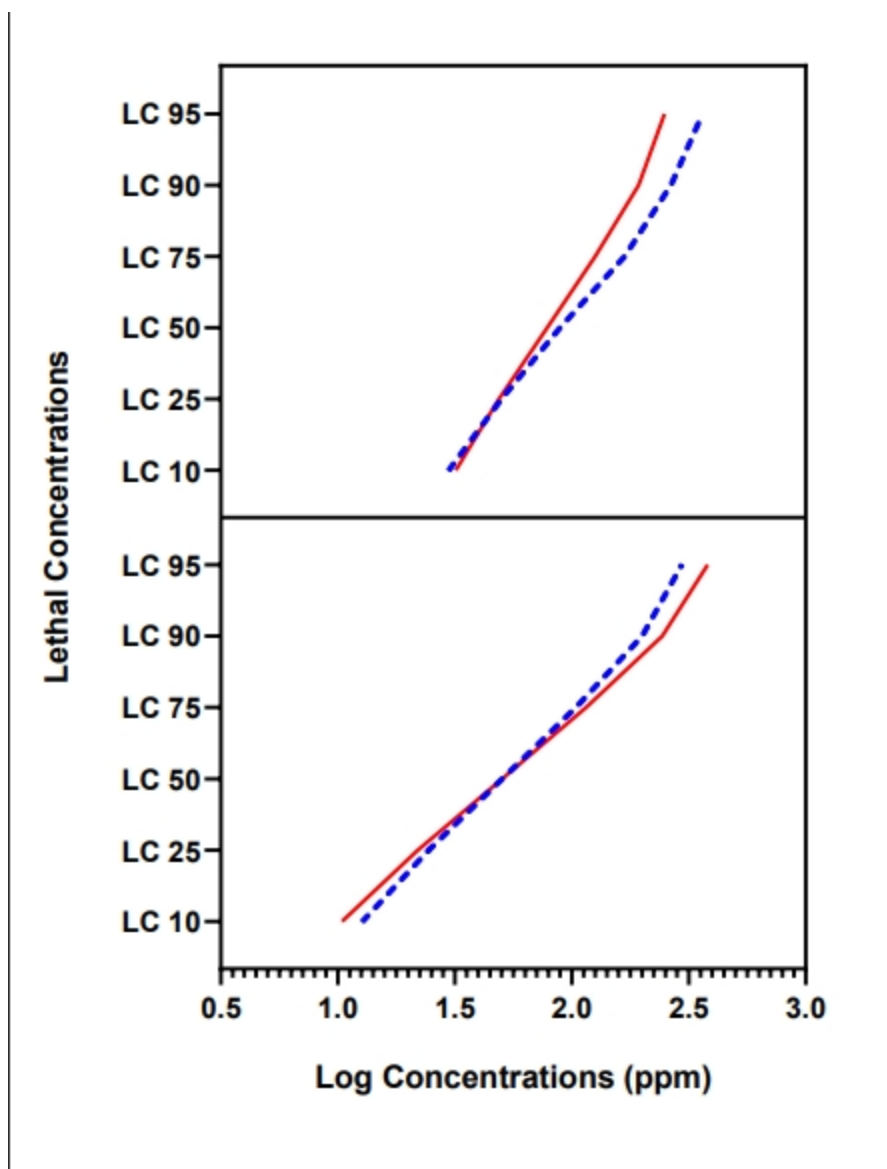


Figure 2

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. (B) microplus* respectively.

Supplementary Files

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