



Original Article

Preliminary Evaluation of the Acaricidal Activity of *Vitex negundo* Linn. (Lagundi) Solvent Extract/Fractions against *Rhipicephalus sanguineus* sensu lato larvae (Latreille 1806)

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ABSTRACT

The acaricidal activity of *Vitex negundo* was determined with the use of hexane, 95% ethanol and water as solvents against larvae of *Rhipicephalus sanguineus* sensu lato. Dried *V. negundo* leaves were sequentially extracted and each filtrate (crude hexane extract or CHE, crude ethanolic fraction or CEF and crude aqueous fraction or CAF) was concentrated with a rotary evaporator. There were different computed concentrations of the solvent/fractions and were used against the larvae through spraying method. Each treatment of the solvent/fractions was replicated three times with at least 10 larvae (ranges from 10-29) and the mortality was observed on 12th and 24th h exposure; death of the larvae were confirmed through prodding with toothbrush bristle. The highest acaricidal activity that has been recorded in this study were 46.09% for 100% CHE, 54.79% for 11.22% CEF and 57.43% for 33.66% CAF; with the LC₅₀ of 16.10% and 31.91% for CEF and CAF, respectively. The results of this study indicated that the acaricidal activity of *V. negundo* extract and fraction against larvae are not effective based on European Agency for the Evaluation of Medicinal Products and as compared to the commercially available acaricide. Perhaps increasing the concentrations of the CEF and CAF could be possibly done for higher mortality of the *R. sanguineus* sensu lato larvae.

KEYWORDS: *acaricidal, In vitro, lagundi, Rhipicephalus sanguineus sensu lato, Vitex negundo*

1 INTRODUCTION

Rhipicephalus sanguineus sensu lato is a three-host tick that feeds primarily on dogs and occasionally on other hosts, including humans in both urban and rural areas (Dantas-Torres, 2008). They are widely distributed around the world and they are known vectors of *Babesia canis* for canine babesiosis, *Ehrlichia canis* for canine ehrlichiosis and *Rickettsia rickettsia* for canine rickettsial disease (Dantas-Torres,

2008; Yabsley *et al.*, 2008). Heavy infestations of its larvae may lead to anemia as they are blood feeders (McTier *et al.*, 2016).

Eradication of *R. sanguineus* sensu lato is often difficult because of the nature of its life cycle (Shaw, Day, Birtles, and Breitschwerdt, 2001). When not in the host, they hide themselves in crevices and dark areas. To combat this type of infestation, there are commercial acaricidal products that are available in the market as spot-on, injectable, sprays, soaps and shampoos which are effective against them. However, the cost of these products is somehow expensive that compliance for the medication and treatment is often discontinued. Aside from the cost, ticks are now developing resistance against various acaricides such as amitraz, BHC/cyclodienes, and organophosphates (Coles and Dryden 2014). Current trends in acaricidal therapy involve the use of herbal extracts, as these natural products would have none or lesser side effects to the host and environment (Anthony, Fyfe, and Smith, 2005). Evaluations on plant extracts for controlling ticks have grown intensely as they have the ability to repel blood-sucking arthropods and people tend to rely its efficacy on treating animal diseases and parasitism (Borges, Sousa, and Barbosa, 2011; McGaw and Eloff, 2008).

One of the plants that has a potential acaricidal effect is the *V. negundo*, which was found to be effective as larvicidal and repellent in mosquitoes like *Anopheles subpictus* and *Culex tritaeniorhynchus* (Kamaraj *et al.*, 2009; Karunamoorthi, Ramanujam, and Rathinasamy, 2008). Singh *et al.*, (2014) studied the effect of *V. negundo* against synthetic pyrethroid resistant *Rhipicephalus microplus* and the results were promising. Currently, there has been no study conducted on the determination of the efficacy of *V. negundo* against larvae of *R. sanguineus* sensu lato. This study can serve as a reference for developing cost efficient, organic based acaricide for clinical use.

2 MATERIALS AND METHODS

Collection and Mass Rearing of Ticks (Mamanao, 2013)

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There were ten dogs used in this study, in which three to five female of *R. sanguineus* sensu lato from each dogs were collected. The ticks were carefully removed from the infested dogs at households in Brgy. Calanggaman, Ubay, Bohol. The collection and culture of ticks were done continuously for two weeks to provide sufficient viable larvae in the scheduled assay.

Identification of the adult tick was performed following the descriptions specified by Soulsby (1982) and Dantas-Torres *et al.*, (2013). In removing the tick from the host, a light pressure grip was applied to avoid damaging or crushing the tick and injuring the host. Collected gravid ticks were placed into individual plastic cup containers lined with a filter paper disc moistened with distilled water to keep it moist. The set-up was covered with a very fine nylon mesh to avoid excessive moisture loss and escape of gravid ticks. Then it was placed in a controlled environment with a temperature of $28\pm 1^\circ\text{C}$ and a relative humidity of $85\pm 5\%$ about two weeks for oviposition. The eggs were maintained in the same condition to allow it to hatch into larvae.

Collection and Extraction of *V. negundo* Leaves

Leaves were collected from barangay Calanggaman, Ubay, Bohol; were washed and cleaned thoroughly and were identified by an expert; they were chopped into small pieces; air dried at room temperature until 90% of its weight was loss and were pulverized. The different solvents used for extraction were hexane, 95% ethanol and water. The extraction procedure as previously described by Fernandez *et al.*, (2013) was followed. The concentrated extract/fractions were stored in a vial and placed in the bench to allow any possible remaining solvents to evaporate; were refrigerated overnight to allow sediments to settle and were transferred to another container through decantation.

Preparation of Varying Concentrations of *V. negundo* Extracts/Fractions and Fipronil

A preliminary study was conducted to determine the LC_{50} of the crude hexane extract (CHE), crude ethanolic extract (CEF) and crude aqueous fraction (CAF). The establishment of the LC_{50} was necessary to approximate the initial concentrations used for the final study. The initial concentrations used for the final study were 100%, 10%, 30% for CHE, CEF and CAF, respectively. The logarithmic method of Guevara and Recio (2005) was used to determine the lower and upper concentrations for the CEF and CAF of *V. negundo* leaves. The CHE was immiscible with distilled water even with the addition of 1% DMSO solution, thus, 100% concentration was used.

Assay for Acaricidal Activity

The experiment was performed with seven treatment groups for CEF and CAF and three treatment groups for CHE, all were replicated three times with at least 10 larvae (ranges from 10-29) on each replicate. Fipronil (T0(+)) was used as positive control and the negative control (T0(-)) was 100% hexane for CHE, 95% ethanol for CEF, and distilled water for CAF; and

five different treatments at different concentrations for CEF and CAF of concentrated *V. negundo*. The concentration used for CHE was only 100% concentration. Whereas for CEF were 7.94%, 8.91%, 10%, 11.22% and 12.59%; while 23.83%, 26.74%, 30%, 33.66% and 37.77% for CAF. In this study, a spray method was employed following that of Mamanao (2013), wherein at least 10 larvae were placed on each Petri plate lined with filter paper in the bottom. The sprayer bottle was filled with 1 ml of the extract/fractions, ensuring that all of it was sprayed all over to the Petri plate with the viable larvae. After exposure of the extracts, the Petri plates were covered with a very fine nylon mesh to provide ventilation and to prevent escape of the larvae. Mortality of the larvae was recorded after 12 and 24 h. To determine whether the larvae were dead or alive, they were prodded gently with a toothbrush bristle. The absence of any movement was considered dead, while those that moved was considered alive (Kusin, Bagot, and Lumain, 2016). The dead larvae were transferred into a white piece of paper for counting and recording.

Phytochemical Analysis

The phytochemical analyses of the extract and fractions as previously described by Senguttuvan *et al.*, (2014) were performed by testing the following secondary metabolites: alkaloids (Mayer's test and Wagner's test), tannins (Ferric Chloride test), saponins (Saponin test), flavonoids (Alkaline Reagent test and Lead acetate test), and terpenoids (Chloroform and sulfuric acid test). A qualitative grading system of the phytochemicals was described in the following manner: (+) positive, (-) traces and (0) undetected.

Statistical Analysis

The experiment was laid out in a Complete Randomized Design (CRD) and the percent mortality was analyzed by one-way ANOVA. The significant means among treatment was then compared using Tukey's Honesty Significant Difference test. Probit analysis was computed to estimate the lethal concentration 50 (LC_{50}) at 95% confidence limit of the *V. negundo* CEF and CAF. The efficacy of the CHE, CEF and CAF was based from the European Agency for the Evaluation of Medicinal Products (EMEA, 2000) wherein $<90\%$ mortality is not effective.

Ethical Considerations

The study was guided by the principles of animal welfare stipulated in the Animal Welfare Act (RA 8485) and Administrative Order No. 45 of the Bureau of the Animal Industry of the Philippines.

3 RESULTS AND DISCUSSION

The acaricidal activity of *V. negundo* CHE, CEF and CAF through spray method are shown in Tables 1, 2, 3 and 4 wherein variation of the efficacy was demonstrated but at dose-dependent manner. For 100% CHE, the highest mean efficacy recorded was at

46.09%. This was not statistically comparable to the commercially available acaricide, which was 100%.

Based on EMEA (2000), the *V. negundo* CHE was not effective.

Table 1. Mean % mortality of *V. negundo* CHE against larvae of *R. sanguineus* sensu lato at 12 and 24 h post-exposure

Treatment	Mean number of larvae	Mean number of dead larvae at 12 h exposure	% Mortality	Mean number of dead larvae at 24 h exposure	% Mortality
T0(-)	11 ± 1.73	0	0% ^a	0	0% ^a
T0(+)	10.33 ± 0.58	10.33 ± 0.58	100% ^c	10.33 ± 0.58	100% ^c
T1	25.6 ± 9.74	5.8 ± 4.32	22.66% ^b	11.8 ± 4.15	46.09% ^b

% mortality with different letters are statistically significant with each other at p= value of 0.05

For *V. negundo* CEF after 12 h exposure, the 95% ethanol as negative control (T0 (-)) was not statistically different between all treatments (7.94% (T1), 8.91% (T2), 10% (T3), 11.22% (T4) and 12.59% (T5)) but statistically different between 0.2% fipronil as positive control (T0 (+)). While, 0.2% fipronil as positive control (T0 (+)) was statistically significant between all treatment groups of CEF. With the 24 h observation, T1-T5 were not statistically different between each other but were statistically different between 0.2% fipronil as positive control (T0 (+)). Based on EMEA (2000), the *V. negundo* CEF was not effective. The LC₅₀ of *V. negundo* CEF was 16.101% based on the Probit analysis at 95% confidence interval.

For *V. negundo* CAF, at 12 h exposure, all treatment groups (23.83% (T1), 26.74% (T2), 30% (T3), 33.66% (T4) and 37.77% (T5)) were not statistically different from each other but not statistically comparable to 0.2% fipronil as positive control (T0 (+)). On the 24 h exposure, all treatment groups from T1-T5 were not statistically different from each other; but T4-T5 between 0.2% fipronil (T0 (+)) was statistically comparable though at lower efficacy. The mean efficacy of 33.66% (T4) and 37.77% (T5) were only 55.25% and 57.43% compared to the 100% mortality of the fipronil, respectively. The LC₅₀ of *V. negundo* CAF was 31.906% based on the result of Probit analysis.

The acaricidal activity of *V. negundo* CHE, CEF, and CAF including 100% hexane (T0 (-)1), 95% ethanol (T0 (-)2) and distilled water (T0 (-)3) as negative controls and 0.2% fipronil (T0 (+)) as positive control against the *R. sanguineus* sensu lato larvae by spray method were significantly different in terms of mortality at 5% level of significance (p= <0.05) at 12 and 24 h exposures (Table 4). Wherein, in both 12 and 24 h exposure, the different treatment groups were not statistically different from each other and the acaricidal activities were not comparable to the commercially available acaricide. The efficacies of CHE, CEF and CAF against *R. sanguineus* sensu lato larvae were 46.09%, 48.22% and 47.89%, respectively. Among the three solvent systems, the 95% ethanol has the highest mean acaricidal activity. The overall efficacy of the three different solvent extract/fractions based on EMEA (2000) was not effective.

Phytochemical analysis was performed to determine the presence of possible secondary metabolites as bioactive compounds that might contributed the

acaricidal activity of the different *V. negundo* extract/fractions (Table 5). Based on the phytochemical results, the *V. negundo* CHE was positive for terpenoids and alkaloids; for *V. negundo* CEF, flavonoids, tannins, alkaloids were present; while for the *V. negundo* CAF, positive reactions to saponins, flavonoids, tannins, and alkaloids were observed.

A blue-black and brownish green discoloration of the test solution indicated the positive reaction for condensed tannins. Stable froth or copious lather at the top of the solution that can be observed for 15 minutes was an indication of saponins. For alkaloids, the Mayer's test was used and positive result was indicated with a cream colored precipitate, while a reddish brown precipitate can be observed in Wagner's test. Terpenoids was observed as a reddish brown precipitate at the test solution when present. And of flavonoids, there were two tests performed: the alkaline reagent test, wherein positive result was indicated with an intense yellow coloration when a few drops of 20% NaOH were added into the solution and became colorless when a dilute acid was added; and lead acetate test, wherein positive result was observed with yellow precipitate.

In this study, the acaricidal efficacy of *V. negundo* CEF against *R. sanguineus* larvae have similarities with Singh *et al.* (2014) wherein its ethanolic extracts showed greater acaricidal activity than the aqueous extracts, but in adult ticks. However, the *V. negundo* CEF efficacy did not meet with the requirement for proposed acaricide set by the European Agency for the Evaluation of Medicinal Products which is >90%. On the other hand, *V. negundo* CAF at concentrations of 33.66% (T4), and 37.77% (T5) were statistically comparable to that of the 0.2% fipronil, with efficacy of 55.25% and 57.43%, respectively; but this acaricidal efficacy was still ineffective based on the EMEA (2000).

The *V. negundo* CHE, CEF and CAF were not highly effective against *R. sanguineus* sensu lato larvae using spray method. This may suggest that a higher concentration should be used to increase larvae mortality. Up to date, there has been no published report against acaricidal activity of *V. negundo* extract/fractions tested against *R. sanguineus* sensu lato larvae. However, several studies on the acaricidal activity against other tick species have been reported. The *V. negundo* leaves ethanolic extract has been

reported to be effective against *Hyalomma anatolicum* (Kamaraj *et al.*, 2009) and synthetic pyrethroids with an LC₅₀ value of 0.011% (Singh *et al.*, 2015); against *R. microplus* with LC₅₀ value of 611.67 ppm (Singh *et al.*, 2014). Moreover, it has been reported

Table 2. Mean % mortality of *V. negundo* CEF against *R. sanguineus* sensu lato at 12 and 24 h exposure

Treatment	Mean number of larvae	Mean number of dead larvae at 12 h exposure	% Mortality	Mean number of dead larvae at 24 h exposure	% Mortality
T0(-)	10.33 ± 0.58	0	0% ^a	0	0% ^a
T0(+)	10.33 ± 0.58	10.33 ± 0.58	100% ^b	10.33 ± 0.58	100% ^c
T1	14.67 ± 2.89	3.67 ± 2.08	25.02% ^a	6.33 ± 2.31	43.15% ^b
T2	11.67 ± 1.53	4 ± 1.73	34.28% ^a	5.33 ± 1.53	45.67% ^b
T3	13 ± 1.00	1.67 ± 1.53	12.85% ^a	6 ± 1.00	46.15% ^b
T4	14 ± 4.36	3.33 ± 1.53	23.79% ^a	7.67 ± 2.89	54.79% ^b
T5	12.33 ± 0.58	4 ± 3.61	32.44% ^a	6.33 ± 2.89	51.34% ^b

% mortality with different letters are statistically significant with each other at p= value of 0.05

Table 3. Mean % mortality of *V. negundo* CAF against larvae of *R. sanguineus* sensu lato at 12 and 24 h exposure

Treatment	Mean number of larvae	Mean number of dead larvae at 12 h exposure	% Mortality	Mean number of dead larvae at 24 h exposure	% Mortality
T0(-)	12.67	0	0% ^a	0	0% ^a
T0(+)	10.33 ± 0.58	10.33 ± 0.58	100% ^c	10.33 ± 0.58	100% ^c
T1	11.33 ± 1.15	1.67 ± 1.15	14.74% ^{ab}	3.67 ± 2.08	32.39% ^{ab}
T2	13 ± 1.00	3.33 ± 0.58	25.62% ^{ab}	6 ± 1.00	46.15% ^{ab}
T3	10.67 ± 0.58	1.33 ± 0.58	40.58% ^{ab}	5.67 ± 2.65	53.14% ^{ab}
T4	12.67 ± 3.79	3 ± 1.00	23.68% ^b	7 ± 1.00	55.25% ^{bc}
T5	15.67 ± 8.14	2.66 ± 0.58	31.91% ^b	8.66 ± 2.52	57.43% ^{bc}

% mortality with different letters are statistically significant with each other at p= value of 0.05

Table 4. Mean % Mortality of *V. negundo* CHE, CEF and CAF against larvae of *R. sanguineus* sensu lato at 12 and 24 h exposure

Treatment	Mean number of larvae	Mean number of dead larvae at 12 h exposure	% Mortality	Mean number of dead larvae at 24 h exposure	% Mortality
T0(-) ₁ 100% Hexane	12.33 ± 1.73	0	0% ^a	0	0% ^a
T0(-) ₂ 95% Ethanol	10.67 ± 0.58	0	0% ^a	0	0% ^a
T0(-) ₃ Distilled Water	12.67 ± 3.06	0	0% ^a	0	0% ^a
T0(+) Fipronil	10.33 ± 0.58	10.33 ± 0.58	100% ^c	10.33 ± 0.58	100% ^c
CHE	25.6 ± 0.58	5.8 ± 0.58	22.66% ^b	11.8 ± 0.58	46.09% ^b
CEF	39.4 ± 3.65	10 ± 2.92	25.38% ^b	19 ± 2.56	48.22% ^b
CAF	38 ± 5.79	7.2 ± 2.59	18.95% ^b	18.2 ± 5.72	47.89% ^b

% mortality with different letters are statistically significant with each other at p= value of 0.05

Table 5. Phytochemical Analysis of the *V. negundo* Extract/ Fractions

Phytochemical substance	Crude hexane extract	Crude ethanolic extract	Crude aqueous extract
Alkaloids	+	++	-
Tannins	0	++	++
Terpenoids	+++	0	0
Flavonoids	0	-	-
Saponins	0	0	++

+++ strongly positive, ++ moderately positive, + weakly positive, - traces, 0 undetected Interpretation (Senguttuvan *et al.*, 2014)

by Vishwanathan and Basavaraju (2010) that the extracts of *V. negundo* possess inhibitory, deterrent or lethal activity on biological agents. Major chemical compound that is found mostly on essential oils of this plant was carvacrol, which is a monoterpene

synthesized from γ-pinene (Kintzios, 2003). The higher levels of carvacrol found on the plant material, the more effective the acaricidal activity (Koc, Oz, Cinbilgel, Ayden, and Cetin, 2013). Though most secondary metabolites are not fully identified in this study, the

presence of more than one secondary metabolite was usually required for acaricidal activity (Arceo-Medina *et al.*, 2016). These secondary metabolites can also act synergistically when used with or in combination with another plant with active ingredient with adjuvant properties (Godara *et al.*, 2015).

Other phytochemical analysis from different studies revealed that *V. negundo* extracts show positive for metabolites such as phenols, irridoids, steroids, phenolic compounds, lignane derivatives, amino acids, fatty acids and aliphatic alcohol which may be contributors for its acaricidal activity (Nirmalkumar, 2007; Nirbhay Kumar Singh *et al.*, 2014). The difference in the bioactive compounds present in the phytochemical analysis can be correlated with several factors like genetic variation and evolution; environmental conditions (storage, edaphic factors, geographic variation, plant diseases and pests, pollution, climate, seasonal variation), and interaction between the two (Aherne, Jiwan, Daly, and O'Brien, 2009; Figueiredo, Barroso, Pedro, and Scheffer, 2008). Among the factors, genetic composition is the main determining factor for the phytochemicals present (Schreiner, 2004). This was supported by Justesen *et al.*, (1998), he had observed the differences in phytochemical compounds between cultivars. On the study of Bautista *et al.*, (2015), it was found out that the *V. negundo* in the Philippines has several morphotypes thus resulting into variations of the result of the phytochemical analysis.

Aside from the difference in phytochemical constituents, most studies of acaricidal activity against larvae employ the larval packet test (LPT) or larval immersion test (LIT) (Fernandes and Freitas 2007; Fernández-Salas *et al.*, 2011; Jacques *et al.*, 2015; Singh *et al.*, 2014). In the current study, spray method was used, this method was selected as it was the mode of application by the fipronil that served as positive control.

The low mortality of the larvae could be associated with the difference in the assay method used in this study. Another factor is the amount of extract sprayed, the filter paper was not impregnated with the extract/fractions and that some larvae are hiding underneath the filter paper. In in vivo set-up, the disadvantages of using the spray method include blockage or clogged of the spray nozzles and under saturation of the surface area sprayed or location (FAO, 2004). During the conduct of the study, clogged nozzle was due to oily consistency of the extract and the presence of some filtrate but it was made certain that the 1ml extract was fully utilized. Further, the effectiveness of the acaricide not only depends on the degree of toxicity on a chemical but also on the quality, quantity and degree of dispersal of the active ingredient (George, Finn, Graham, and Sparagano, 2014).

4 CONCLUSION

The efficiency of hexane, 95% ethanol and distilled water as solvents in drawing out the possible secondary

metabolites as bioactive compounds of *V. negundo* were almost similar. However, among the three solvents, 95% ethanol has the highest efficacy. Also, The *V. negundo* CHE, CEF and CAF were inefficient as an alternative acaricide against *R. sanguineus* sensu lato larvae using spray method based on the efficacy requirement set by the European Agency for the evaluation of Medicinal Products. In addition, it was found out that the LC₅₀ of the *V. negundo* CHE and CAF against *R. sanguineus* sensu lato larvae were 16.101% and 31.906%, respectively at 24 h exposure. The phytochemicals present in the study for *V. negundo* CHE were alkaloids and terpenoids, for *V. negundo* CEF alkaloids, tannins and flavonoids, and for *V. negundo* CAF were saponins, alkaloids, tannins and flavonoids.

RECOMMENDATIONS

Different methods in the application of the extract may be employed in acaricidal assay, specifically larval packet test or larval immersion test. Further, it would be beneficial to increase the dosage rates or concentrations of the extract/fractions against larvae of *R. sanguineus* that may result to a higher mortality; and for narrower margin of error of the results, increase the number of samples per replicate. Moreover, the *V. negundo* extract/fractions may be used for other ectoparasites of other domestic animals. Finally, conduct quantitative analysis of the bioactive components of the *V. negundo* extract/fractions to support the efficacy of the extracts; and, isolation and characterization of the bioactive compounds are important for future reference.

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