

Bioactivity of *Annona montana* Macfad extracts on the black cowpea aphid (*Aphis craccivora* Koch)

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ABSTRACT

This study aimed to verify the insecticidal activity and determine the LC₅₀ of hydroethanolic extracts from *Annona montana* leaves and seeds on the black aphid, *Aphis craccivora*. The experiment was conducted in a greenhouse. *Annona montana* leaf and seed extracts were obtained by solvent extraction using 70% ethanol. The following spray treatments were applied on the black aphid nymphs: Hydroethanol (HE) extracts from leaves at concentrations of 2.5; 5; 10; 15%, and EH extracts from seeds at 0.5; 1.0; 1.5; 2%; Neemax[®] and distilled water (control). Results showed the presence of insecticidal effect of both extracts on *A. craccivora* nymphs. The HE from leaves was more efficient at the concentration of 15%, with 78% mortality of nymphs. In the case of HE from seeds, the largest mortality was obtained at the concentration of 2%. The acute toxicity of *A. montana* extracts on *A. craccivora* nymphs was 7.69 for HE from leaves and 0.55 for HE from seeds. Therefore both extracts of *A. montana* may be indicated to the control of the agricultural pest (*A. craccivora*).

Key words: Annonaceae; Aphididae; botanical insecticide

Bioatividade de extratos de *Annona montana* Macfad sobre o pulgão-preto do feijão-caupi (*Aphis craccivora* Koch)

RESUMO

Objetivou-se verificar a atividade inseticida e determinar a CL₅₀ dos extratos hidroetanólicos de folha e semente de *Annona montana*, sobre o pulgão-preto, *Aphis craccivora*. O experimento foi realizado em casa de vegetação. Os extratos de folhas e sementes de *A. montana*, foram obtidos por extração com solvente etanol a 70%. Sobre as ninfas do pulgão-preto foram aplicados via pulverização os tratamentos: Extratos hidroetanólico (EH) de folhas na concentração de 2,5; 5; 10; 15% e, (EH) de sementes a 0,5; 1,0; 1,5; 2%; Neemax[®] e água destilada (testemunha). Os resultados apontaram o efeito inseticida de ambos extratos sobre as ninfas de *A. craccivora*. O EH das folhas apresentou maior eficiência na concentração a 15%, com 78% de mortalidade das ninfas, para o EH das sementes o maior índice de mortalidade de ninfas foi obtido com a concentração de 2%. A toxicidade aguda dos extratos de *A. montana*, sobre as ninfas de *A. craccivora*, foram de 7,69 para os EH de folhas e de 0,55 para os EH de sementes. Portanto ambos os extratos de *A. montana* podem ser indicados no controle da praga agrícola (*A. craccivora*).

Palavras-chave: Annonaceae; Aphididae; inseticida botânico

Introduction

Several plants can cause some kind of damage to insect physiology due to the formation of compounds and intermediate and final products, especially in their secondary metabolism (Turchen et al. 2016). When extracted, these compounds may have effects such as repellency, feeding and oviposition deterrence, sterilization, metabolic blockade and interference in development (Castillo-Sánchez et al., 2010; Moore et al., 2013).

Annonaceae stands out among plant families with potential for production of compounds with high insecticidal activity produced by their secondary metabolism (Ribeiro et al., 2016). Presence of alkaloids and formation of acetogenins derived from long fatty acid chains are among the main bioactive constituents found in specific genres of Annonaceae. They have important biological activities such as cytotoxic, antitumor, insecticide, vermicide, abortive, antimicrobial, and immunosuppressant activities, among others (Nascimento et al., 2003; Silva et al., 2007b).

Acetogenins are distributed throughout the plant, but the highest concentration happens in the seeds (Bermejo et al., 2005). They act as potent inhibitors of the respiratory chain, affecting the mitochondrial complex I, causing respiratory chain blocking through the inhibition of the NADH ubiquinone oxidoreductase, an essential enzyme in complex I. This prevents oxidative phosphorylation, directly affecting the electron transport in the cell mitochondria and causing a decrease in ATP levels, leading to apoptosis (Bermejo et al., 2005; Rattan 2010).

Because they have diverse structures and powerful cytotoxic properties, acetogenins are promising in the development of new agricultural insecticides (Isman & Seffrin, 2014). According to Isman (2006), these compounds act as stomach poisons, and for this reason they are effective to kill chewing insects such as lepidopteran caterpillars and the Colorado potato beetle (*Leptinotarsa decemlineata* (Say) (Coleoptera: Chrysomelidae)). However, González-Coloma et al. (2002) observed that the effects of a particular type of acetogenins depend on the species/cells and structures. Thus, acetogenin structure-activity studies should be directed to the target.

Cowpea bean (*Vigna unguiculata* (L.) Walp) is one of the main crops cultivated in the Northern region, especially in family-based farming. Among the many insect pests that attack this crop in the Amazon region, there is the black aphid, *Aphis craccivora* Koch (Hemiptera: Aphididae) (Bandeira et al., 2015). This aphid usually starts colonizing the terminal buds and petioles of the young leaves of its host. It is an important plague mainly because the insect injects toxins and can be transmitters of several viruses (Moraes & Bleicher, 2007, Das et al., 2008). Furthermore, the high feeding capacity of this sap-sucking pest causes shriveling of leaves and deformation of shoots. This event also requires the elimination of large amounts of sugary liquid or "honeydew," which serves as substrate for development of sooty mold. These fungi, in turn, cover the leaves and reduce the photosynthetic capacity of the plant (Silva & Bleicher, 2010).

Studies report the insecticidal and/or anti-feeding properties of various Annonaceae species (Krinski & Massaroli, 2014). However, few studies address the insecticide effect of *A. Montana* extracts. Cowpea is commonly cultivated in subsistence agriculture. The use of alternative pest-control methods with less harmful effects of agriculture on the environment is important. Thus, the objective of this study was to verify the insecticidal activity and determine the LC₅₀ hydroethanolic extracts from leaves and seeds of *Annona montana* Macfad on the black cowpea aphid.

Material and Methods

The study was conducted during the months of January and February 2013 in a greenhouse at the Center for Agricultural Sciences (CCA) and the Plant Protection Laboratory at Cauamé Campus, Federal University of Roraima (UFRR), located in the rural area of Monte Cristo, in Boa Vista city, Roraima.

Black aphids were obtained from cowpea planting in an experimental area in the CCA/UFRR. After collection, the aphids were kept in a cage (1.6 x 3.0 x 2.0m) coated with antiaphid screen, where the species started to proliferate in cowpea plants of the UFRR - Green grain variety, kept in vessels. During the survey period, a digital termohigrographic recorded the daily variation in temperature and relative humidity, with an average of 30.52 ± 5° C and 70% relative humidity, respectively.

Plant material (leaves and fruits) of *A. montana* were collected from plants located in the Cauamé Campus and taken to the Plant Protection Laboratory (CCA-UFRR) where the extract obtention process was carried out. Identification of the botanical material was based on morphological characters and was done by the Embrapa-RR researcher Dr. Edvan Alves Chagas.

Leaves were washed in running water and disinfested with sodium hypochlorite at 2% for 20 minutes, to eliminate microorganisms on leaf surface. Thereafter, the material was rinsed in distilled water to remove the excess of hypochlorite. Seeds were taken from the collected mature fruits and washed to remove any residual pulp.

Both materials were spread on paper towels and left for 48 hours to reduce the humidity. After 48 hours, the material was placed in paper bags and put into forced air circulating oven, where they remained for 72 hours at 45° C.

After disinfestation and drying of leaves and seeds, the material was separately submitted to trituration in a Willey-type mill to obtain leaf and seed powder. The powder was diluted in 70% alcohol at the ratio of 30g of powder to 70 ml of solvent, and subjected to stirring at 14000 rpm for two periods of five minutes with a three-minute interval.

Solutions obtained from the leaves and seeds were placed in Erlenmeyer flasks, where remained still for seven days. After this period, solutions were filtered in funnel with aid of a vacuum pump. For removal of the alcohol present in the solution, the filtered material was subject to rotoevaporation at 45° C, obtaining the *A. montana* leaf and seed extracts.

The hydroethanolic extract from leaves and seeds were subjected to dilution (addition of distilled water), to obtain

concentrations of 15% (v/v); 10% (v/v); 5% (v/v) and 2.5% (v/v) to the leaf extracts and dilutions of 2% (v/v); 1.55% (v/v); 1% (v/v) and 0.56% (v/v) of the seed extracts.

As control treatment, the commercial product Neemax® - (0.12% of azadirachtin) was used to prepare a 100 mL solution as recommended by the manufacturer, 1L of product to 100L of water. Distilled water was used as absolute control. It is worth noting that *A. montana* leaf and seed extract doses were tested in independent trials and with the same control and witness treatment. The experimental design used in each trial was randomized, with six treatments and six replications.

The test in semi-field conditions was conducted with nymphs in 1.6 x 3.0 x 2.0 m cages coated with antiaphid screen. Initially, two cowpea seeds of the UFRR- Green grain variety were planted in plastic 300 mL cups containing substrate composed of 50% of sifted soil, 30% of earthworm humus, and 20% Organoamazon® commercial substrate. Thinning was carried out seven days after emergence (D.A.E.) of plants, leaving one plant per pot (experimental unit).

At 20 D.A.E., plants were infested with the aid of a brush. Six *A. craccivora* adult females were placed in each plant and after 48 hours, the adult females were taken off. With the aid of a 10x magnifying glass, the nymphs born were counted; the standard of 20 nymphs per plant was adopted. Then, treatments were applied.

Treatments were applied with the aid of a Strong® high-pressure vaporizing spray (capacity of 1.5 liters), in high volume, with full coverage of the leaf surface. Pulverization was conducted after 17h00m in order to avoid the interference of temperature and sunlight with the effect of treatments. Evaluation was conducted two days after treatment application; living aphids were counted and aphids were considered alive when they moved after a light touch with the brush.

Based on the verification of homogeneity of variance by Hartley test, data were transformed into percentage and later, arcsin transformed $\sqrt{x}/100$. All data were subjected to variance analysis through F test and the means were compared by Tukey test at 5% probability of error. The SISVAR software was used to perform the analysis.

The Probit method was used to determine the LC_{50} of extracts on the nymphs in a 95% confidence interval. This analysis was carried out in the BioStat statistical software. Values were subjected to Probit method previously corrected according to the formula of relative efficiency according to Abbott (1925).

Results and Discussion

We observed that all *A. montana* leaf extract dosages showed insecticidal effect on *A. craccivora* nymphs with statistical difference from the witness treatment. The 15% HE dosage was the one that caused greater insecticidal effect on black aphid nymphs, with 78.04% mortality; these values were not significantly different from the azadirachtin-based control treatment (Table 1).

Annona montana seed extracts in all concentrations showed insecticidal activity on nymphs with significantly different values from the absolute control. The concentrations

Table 1. Average mortality (%) of *A. craccivora* nymphs 48 hours after application of different concentrations of *A. montana* leaf extract and mortality of the control treatment.

Treatment	Mortality (%)	Corrected mortality (%) ER - Abbott (1925)
Witness	3.37 ± 1.34 d	0
HE leaf 2.5%	29.70 ± 4.09 c	27.25
HE leaf 5%	52.52 ± 2.74 b	50.86
HE leaf 10%	52.92 ± 3.49 b	51.28
HE leaf 15%	78.04 ± 2.94 a	77.27
Control	89.78 ± 2.93 a	89.42
CV.	14.37	

Means followed by the same letter do not differ by Tukey test ($P \leq 0.05$); Original data, results from the arcsin transformation $\sqrt{x}/100$.

Table 2. Average mortality (%) of *A. craccivora* nymphs 48 hours after application of different concentrations *A. montana* seed extract, and mortality of the control treatment.

Treatment	Mortality (%)	Corrected mortality (%) ER - Abbott (1925)
Witness	4.55 ± 1.56 c	0
HE seed 0.56%	41.23 ± 3.65 b	38.42
HE seed 1.0%	90.92 ± 2.49 a	90.49
HE seed 1.55%	92.58 ± 3.43 a	92.23
HE seed 2%	96.79 ± 1.65a	96.64
Control	95.01 ± 2.06 a	94.77
CV.	14.16	

Means followed by the same letter do not differ by Tukey test ($P \leq 0.05$); Original data, results from the arcsin transformation $\sqrt{x}/100$.

of 1.0%, 1.55%, 2.0% and the control treatment increased the percentage of mortality of nymphs.

Annona montana seed extract at 2% caused 96.64% of the adjusted mortality, while the application of HE from leaves at 15% resulted in 77.27% of death of nymphs. The greatest efficiency of seed extracts at lower concentrations may have occurred due to the higher amount of acetogenins and/or alkaloids. It is suggested that the allocation of resources stored for the biosynthesis of alkaloids and acetogenins occurs at specific stages to assist the development of the plant. Acetogenin formation seems to occur in plant embryos, during embryogenesis, and can be distributed in different plant structures. In contrast, alkaloids are typically present in leaves and seeds (Gonzalez-Esquinca et al., 2014).

Alkaloids such as anonina and muricana, which are found in *Annona muricata* L., are used in insecticide production. Acetogenins are distributed throughout the plant, but the highest concentration happens in the seeds (Bermejo et al., 2005). Colom et al. (2008) found that acetogenins isolated from *Annona cherimola* Mill seeds (Squamocin, Molvizarin, Itrabin, Almuñequin) have different toxicity levels and are more toxic than acetogenins isolated from *A. montana* leaves (Annonacin-A, Densicomacin, cis-anonacin-10-one, Murihexocin-A, Cherimolin-1) when they are applied in *Oncopeltus fasciatus* Dallas, Hemiptera: Lygaeidae.

Ethanol extract from *Annona squamosa* L. leaves at 25%, caused a mortality of 30.43% of the aphid that attacks sugarcane (*Ceratovacuna lanigera* Zehnter, Hemiptera: Aphididae), 48 hours after the first topical application of extracts and a mortality of 43.48% 48 hours after the second application (Patil & Chavan, 2009). Considering the data presented in Table 1, the results for *A. montana* extract are more significant because lower concentration caused higher aphid mortality, with

77.27% of lethal effectiveness. This may possibly occur due to higher concentration of pesticide substances in *A. montana*.

Research on the effect of the methanol extract from *Annona coriacea* Mart seeds on the tomato leafminer (*Tuta absoluta* Meyrick, Lepidoptera: Gelechiidae), high levels of insect mortality were observed. At the concentration of 0.5%, the result obtained without correcting efficiency was 86.4%, and in the case of the extract at 1%, mortality was 100% (Silva et al., 2007a). These results resemble the ones shown in Table 2 in the present study, wherein *A. montana* seed extract at 1.0% promoted 90.92% of aphid death.

The effect observed on the *A. craccivora* after application of HE from *A. montana* seed extract was darkening and drying of the insects. The symptoms were observed at all concentrations tested. Krinski & Massaroli (2014) reported that the application of *Annona mucosa* Jacq. seed extract caused tremors and later death of *Tibraca limbativentris* (Hemiptera: Pentatomidae) nymphs. This suggests that death happened because of excessive acetylcholine in the body of the insects caused by acetylcholinesterase inhibition.

The dryness of *A. craccivora* nymphs possibly occurred as a result of starvation, due to inhibition or reduction of the food need of aphids. The action of acetogenins, by contact or ingestion, inhibits the mitochondrial complex I and interferes with the sodium channels which are regulated by voltage, changing the sodium and potassium balance. This prevents normal nerve transmission and causes paralysis followed by death ("Knockdown") (Colom et al., 2007).

Lin et al. (2009) observed similar effects in whitefly nymphs, *Bemisia tabaci* Gennadius (Homoptera: Aleyrodidae) after spraying *A. squamosa* seed oil. Shrinking and dryness effect were observed in the insect body. The most effective oil concentrations were 0.5 and 0.25%. The results were evaluated at five and ten days after application of the treatment and efficiency was progressive, with satisfactory results after ten days of application.

Median lethal concentrations (LC_{50}) calculated from the mean insect mortality at concentrations of 2.5; 5.0; 10; and

15% of HE from leaves and 0.56; 1.0; 1.55; and 2.0% of HE from seeds of *A. montana*, at 48 h time interval, were 7.69% and 0.55%, respectively, with 95% confidence interval (Figure 1). Acute toxicity of *A. montana* leaf and seed extracts on *A. craccivora* nymphs is shown in Table 3.

The LC_{50} of *A. montana* leaf extract, although high (7.69%), can be considered as an alternative for small producers, such as horticulturists practicing subsistence agriculture, in view of the easy availability of raw materials (leaves).

Water and ethanol extracts of *A. squamosa* leaf were efficient in controlling *B. tabaci* (whiteflies), with a result above 95% of egg mortality, with LC_{50} of 0.36% for aqueous extracts and LC_{50} of 2.71 mg/mL for the ethanolic extract. Effectiveness in the case of whitefly nymphs was observed only in the ethanol extract, which had 100% of efficiency, with LC_{50} of 2.66 mg mL⁻¹, or an average of 2.66% of the ethanolic extract (Cruz-Estrada et al., 2013).

In a survey conducted by Dadang (2011) using methanol extracts of *A. squamosa* seeds to control the horticultural pest *Cricidolomia pavonana* Fabricius (Lepidoptera: Crambidae). The author found that the effect of topical application of the extract promoted death of 100% of the larvae and determined LC_{50} of 0.52 %, which is close to the value found in the present study, in Table 3, wherein the acute toxicity of hydroethanolic extract of *A. montana* seeds was 0.55%, but determined as the effect on aphids and with a different solvent.

Ethanol extracts (0.5 p/v) of *ata* (*Annona squamosa* L.) and *atemóia* (*Annona cherimola* Mill. x *A. squamosa* L.) seeds formulated from seeds after 0; 5 and 6 years of storage reduced the populations of aphids with efficiency of 96.14%, 98.16% and 97.32%, respectively (Rabelo & Bleicher, 2014). This demonstrates the possibility of storing seeds for prolonged periods without losing insecticidal properties.

Table 3. Acute toxicity of *A. montana* leaf and seed extract on *A. craccivora* nymphs. (Median Lethal Concentration – LC_{50}).

Extract	LC_{50} (%)	Confidence interval (CI - 95%)	
		Lower	Higher
Leaf	7.6897	6.1555	9.2238
Seed	0.5477	0.4381	0.6574

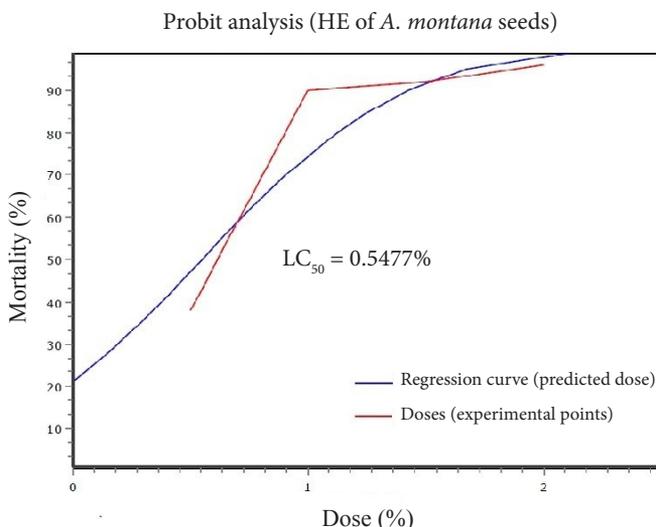
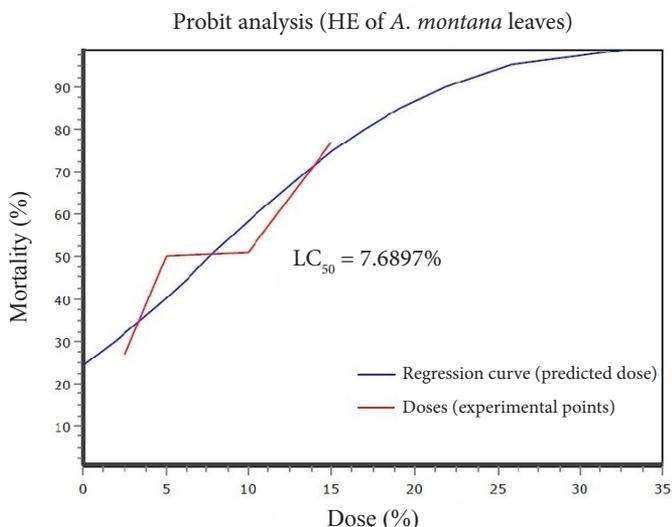


Figure 1. Median Lethal Concentration (LC_{50}) of hydroethanol extract from *A. montana* leaves (A) and LC_{50} of the hydroethanol extract from *A. montana* seeds (B) on *A. craccivora* nymphs (mortality values are corrected by Abbott's formula).

The present results are of great relevance to biopesticide studies. The use of hydroethanolic extract from *A. montana* seeds was effective for the control of *A. craccivora*, with high mortality rates obtained with low concentrations of the extract. There are few studies carried out with *A. montana*, and the results show the potential of the species for use as natural insecticide.

Conclusions

The hydroethanolic extract from *A. montana* leaves and showed insecticidal activity against *A. Craccivora* nymphs. However, the concentration required to obtain satisfactory control with the leaf extract is high, which limits the practical application in small scales.

The highly significant insecticidal effect of hydroethanolic extract from *A. montana* seeds suggests increased activity and/or concentration of agents with insecticidal properties in the seeds. Performing phytochemical analysis and fractionation of the chemical components is necessary to identify the active substances acting on the insects. Such studies can offer new possibilities for formulations to the effective and economical control *A. craccivora*.

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