

Rollinia mucosa (Jacq.) Baillon (Annonaceae) active metabolites as alternative biocontrol agents against the lace bug *Corythucha gossypii* (Fabricius): an insect pest.

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Abstract

The lace bug, *Corythucha gossypii* (Fabricius) is a serious pest affecting over 24 wild and commercially important plant species of the families Annonaceae, Passifloraceae, Caricaceae, Euphorbiaceae, and Solanaceae. Thus far, commercial insecticides, such as 0.1 % Dimethoate and 0.1 % Imidacloprid have shown effectiveness against this insect, but no botanical pesticides are available to control this bug. In the present study, a *Rollinia mucosa* (Jacq.) Baillon ethanol extract was evaluated as a biological control agent against the lace bug. Through a toxicity assay involving *Artemia salina*, the median lethal concentration (LC₅₀) of a raw ethanol extract of *R. mucosa* seeds was determined, as well as that of its Acetogenin (F1) and Alkaloid (F2) fractions; these LC₅₀ were 0.184, 0.082, and 0.0493 $\mu\text{g}/\text{mL}$, respectively. In addition, with an insecticide assay on lace bug nymphs, a mortality percentage of 86.67 % at 5 $\mu\text{g}/\text{mL}$ after 72h was observed. These data demonstrate that the *R. mucosa* seed extract is highly active. Further chemical characterization studies revealed that the main active metabolites contributing to extract activity were acetogenins and alkaloids.

Keywords: Acetogenins; Lace bug *Corythucha gossypii*; Isoquinoline alkaloids; Biocontrol; *Rollinia mucosa*

Introduction

The lace bug, *Corythucha gossypii* (Fabricius), can be found across most of the American continent, ranging from southern United States and Mexico to Ecuador, as well as in the West Indies (Varón *et al.* 2010). The lace bug has the widest plan host range among foliage-eating insects, having been reported in sour soup or guanábana (*Annona muricata*), passion fruit (*Passiflora edulis*), and granadilla (*Passiflora ligularis*). In addition, certain perennial crops, such as papaya (*Carica papaya*), and annual crops like yucca (*Manihot esculenta*), sweet potato (*Ipomoea batata*), aubergine

(*Solanum melongena*), and hot pepper (*Capsicum baccatum*) are also affected by this insect (Guidoti *et al.* 2015; Varón *et al.* 2010).

Lace bugs feed on the underside of leaves and their damage is characterized by premature leaf senescence, followed by focalized yellowing on the leaf blade, which can lead to complete leaf bleaching. These events can reduce plant vigor, thus decreasing fruit production or preventing its formation (Varón *et al.* 2010). Lace bug-directed insecticides of the neonicotinoid and the methyl-carbamate types, with long residual effect, are commonly used (Nair & Braman, 2012).

The use of chemical insecticides in pest control programs has triggered secondary pest outbreaks and has been lethal to non-target organisms. Furthermore, increasing insecticide doses, rises the chance of collateral, negative effects on human health, due to the long-term persistence of insecticide residues and their inherent toxicity. In addition, contamination has been reported in water, in the atmosphere, and in the environment as a whole (Ratnadass *et al.* 2012; Savary *et al.* 2012). As an alternative to insecticide use, integrated pest management (IPM) strategies are being implemented, and they include, alternate crops with repellent plants, crop rotation, intercalated crops, and biopesticide use (Ratnadass *et al.* 2012).

Plant-derived extracts have been extensively studied to develop alternatives to conventional insecticides. To this end, species of the Meliaceae, Rutaceae, Asteraceae, Labiateae, Piperaceae, and Annonaceae families have been studied for their bioactivity (De Cássia Seffrin *et al.* 2010).

The Annonaceae family includes trees and shrubs exclusively found in the tropical and subtropical regions of America, Africa, and Asia, and it comprises 130 genera and 2 300 species (Castillo *et al.* 2010; Santos Lima *et al.* 2010). Numerous bioactive substances of diverse chemical nature are present in leaves, roots, fruits, and seeds of Annonaceae plant species. In addition, acetogenin and alkaloid bioactivities are associated with insecticide and pesticide effects (Galvis *et al.* 2010; Krinski *et al.* 2014; Solís *et al.* 2010). The Colombian territory harbors a large number of Annonaceae species, distributed in the following regions: Amazon (54 %), Pacific (27.5 %), and Andean (27 %) (Murillo, 2001).

Rollinia mucosa, commonly called Amazon custard apple (anón), is a wild fruit native of the Amazon (Fonseca *et al.* 2012; Rodrigues *et al.* 2010). Previous research done on this fruit showed that its aporphine alkaloids exhibit antimicrobial and fungicidal activities (Caetano & Dadoun, 1987).

Furthermore, the acetogenins present in this plant have a cytotoxic effect against solid tumors (Chávez *et al.* 1998; Shi *et al.* 1997). To the best of our knowledge, the insecticidal activity of *R. mucosa* seed extracts has not yet been studied and reported. Based on previous studies of the Annonaceae family, we set out to evaluate *R. mucosa* seed ethanol extract to determine its potential use as a bio-pesticide against the lace bug and to identify the main active metabolites responsible for this activity.

Materials and methods

Chemical reagents: TEGO 51, Chloroform, and DMSO were purchased from Merck (Darmstadt, Germany). Ethanol (EtOH), Methanol (MeOH), and Formic Acid were purchased from Sigma-Aldrich (St. Louis Missouri, USA). Acetonitrile (HPLC grade), *n*-Hexane and Dichloromethane were purchased from J.T. Baker Chemicals (Center Valley PA, USA). Sea salt was purchased from Proquimel (Santa Rosa del Cabal, Colombia). Bifenthrin was purchased from Bayer Crop Science (Monheim am Rhein, Germany). Brine shrimp (*Artemia salina*) was gotten from San Francisco Bay Brand (Newark CA, USA). Finally, *Corythucha gossypii* at III, IV, and V nymphal stages were obtained from Hacienda Calamar, Pereira, Colombia.

Standards: Stock solutions of Bullatacin (1) (C₃₇H₆₆O₇; molecular weight 622.928 g/mol), and Papaverine (2) (C₂₀H₂₁NO₄; molecular weight 339.39 g/mol) were prepared in acetonitrile to a concentration of 100 µg/mL.

R. mucosa seed preparation

Plant material: *R. mucosa* specimens were collected in the municipality of Santo Domingo, department of Caquetá, southern Colombia. Species status was confirmed at the Herbarium of the Universidad de la Amazonia (HUAZ) with voucher number 1525.

Pre-treatment: Seeds from the collected *R. mucosa* fruits were washed with TEGO 51 and dried at 37 °C for 72 h. Dry material was then ground and degreased by means of the Soxhlet technique using hexane (1:4 w/v) for 24 h (Castro *et al.* 2010).

Chemical extraction from *R. mucosa* seeds and analysis

Obtaining the seed extract: Dry, ground seed material was subjected to passive maceration using ethanol as solvent, at a 1:4 sample-solvent ratio (Castro *et al.* 2010; Chen *et al.* 2012; McLaughlin, 2008). Maceration took

place at room temperature for one week, with gentle stirring of the macerating material once a day. The Extract was then filtered and concentrated in a rotary flash evaporator and kept at -4 °C.

Chemical analysis: The chemical characterization of the *R. mucosa* active metabolites was carried out with a liquid-liquid extraction. Starting from a raw ethanolic extract, two fractions were obtained and designated as F1 and F2. F1 corresponds to the acetogenin fraction, obtained from an amount of the raw extract, which is diluted in an aqueous solution of dichloromethane. The organic phase of the resulting F1 solution was then diluted using a methanol-hexane solution. Only the phase obtained with methanol was retained (Castro *et al.* 2010). The F2 fraction, corresponding to the alkaloid fraction, was obtained from a volume of the raw extract diluted with an aqueous solution of 50 % ethanol in a 1:10 ratio. Subsequently, the necessary volume of a hydrochloric acid solution was added until the solution's pH was 4. Then, the necessary volume of an ammonium hydroxide solution was added until reaching a pH of 8. Afterwards some chloroform is added to the remaining phase which splits; fraction F2 goes to the chloroform phase (Rakotondramasy *et al.* 2008).

Profiling by HPLC: HPLC was performed on a Jasco 2000 Plus chromatography system (Jasco International Co Ltd, Tokyo, Japan), consisting of a quaternary gradient pump (PU-2089 Plus), a smart auto sampler (AS-2059 Plus), a column oven (CO-2065 Plus), and a UV-Vis detector with diode array (MD-2015 Plus). For the acetogenin fraction, an isocratic system was employed with a mobile phase consisting of water (A) and an acetonitrile (B) (8:92) at a flow rate of 0.4 mL/min for 10 minutes, and detection set at 220 nm. Profiling was based on a UV-VIS spectrum obtained by HPLC from a Bullatacin standard (Castro *et al.* 2010). For the isoquinoline alkaloid separation, an elution by binary gradient system was conducted with a mobile phase consisting of 0.1 % Formic Acid pH 3.5 (A) and Acetonitrile (B). The analysis was initiated using 70 % of A and progressed until reaching 60 % of B for 35 minutes with at a flow rate of 0.5 mL/min and a detection wavelength set at 270 nm (Ghanavi *et al.* 2013). Profiling was based on a UV-VIS spectrum obtained by HPLC from a Papeverine standard.

Bioassays

Toxic activity: A toxicity assay of the raw ethanol extract, acetogenin fraction (F1), and alkaloid fraction (F2) were conducted on brine shrimp, *A. salina* following a modified McLaughlin method (McLaughlin, 2008). Stock solutions of the raw ethanol extract and the F1 and F2 fractions were prepared

to a concentration of 100 $\mu\text{g}/\text{mL}$, and a mortality test was conducted in triplicate exposing *A. salina* to increasing concentrations of seed extract, ranging from 0.05 to 1 $\mu\text{g}/\text{mL}$ (*i.e.* 0.05, 0.1, 0.2, 0.4, 0.6, 0.8, and 1.0 $\mu\text{g}/\text{mL}$). Artificial seawater was used as negative control, and anhydrous ethanol was used as positive control.

Mortality was assessed after 24 h and reported in terms of the Mean Lethal Concentration (LC_{50}), that is the required concentration ($\mu\text{g}/\text{mL}$) to kill half of the experimental subjects in a given time frame. The data were subjected to Probit analysis.

Insecticidal activity against the lace bug: An *in vitro* assay was implemented with *C. gossypii* at III, IV, and V nymphal stages; on average in a lapse of three days individuals move from one nymphal stage to the next. Each experimental unit consisted of a plastic box with a lid and a ventilation system, one leaf of *A. muricata*, and five *C. gossypii* individuals of the three nymphal stages. The nymphs and the *A. muricata* leaves were collected at Hacienda Calamar, Pereira, Colombia.

Seed extract solutions of concentrations 5, 10, 25, 50, and 100 $\mu\text{g}/\text{mL}$, prepared from a stock solution at 1000 $\mu\text{g}/\text{mL}$, were sprayed over the content of the boxes; making sure that the underside of the *A. muricata* leaves was impregnated previous to placing five nymphs in each experimental unit.

Nymph mortality was assessed 24 h, 48 h, and 72 h after exposure to seed extracts. The negative control consisted of nymphs without any extract exposure, and nymphs exposed to Bifenthrin were used as positive control. In addition, a group of nymphs were exposed to an aqueous ethanol solution to rule out any effect of the ethanol (contained in the extracts) on the insects.

Results and discussion

The average yield of the degreased *R. mucosa* seed ethanol extract was 1.78 %, and those of the enriched acetogenin (F1) and alkaloid (F2) fractions were of 1.19 % and 0.38 %, respectively.

Chemical analysis: The Chemical characterization results of the *R. mucosa* seed extract made by HPLC is presented in **Fig. 1A**, **Fig. 1B**, and **Fig. 1C**. The chromatographic profile of the raw ethanol extract revealed six peaks (2-5, 7, and 9 in **Fig. 1A**). Likely corresponding to acetogenin compounds. This was ascertained thanks to their UV-VIS spectrum, detecting peaks of maximum absorption in the 200-220 nm range (Avelar Lage, 2011).

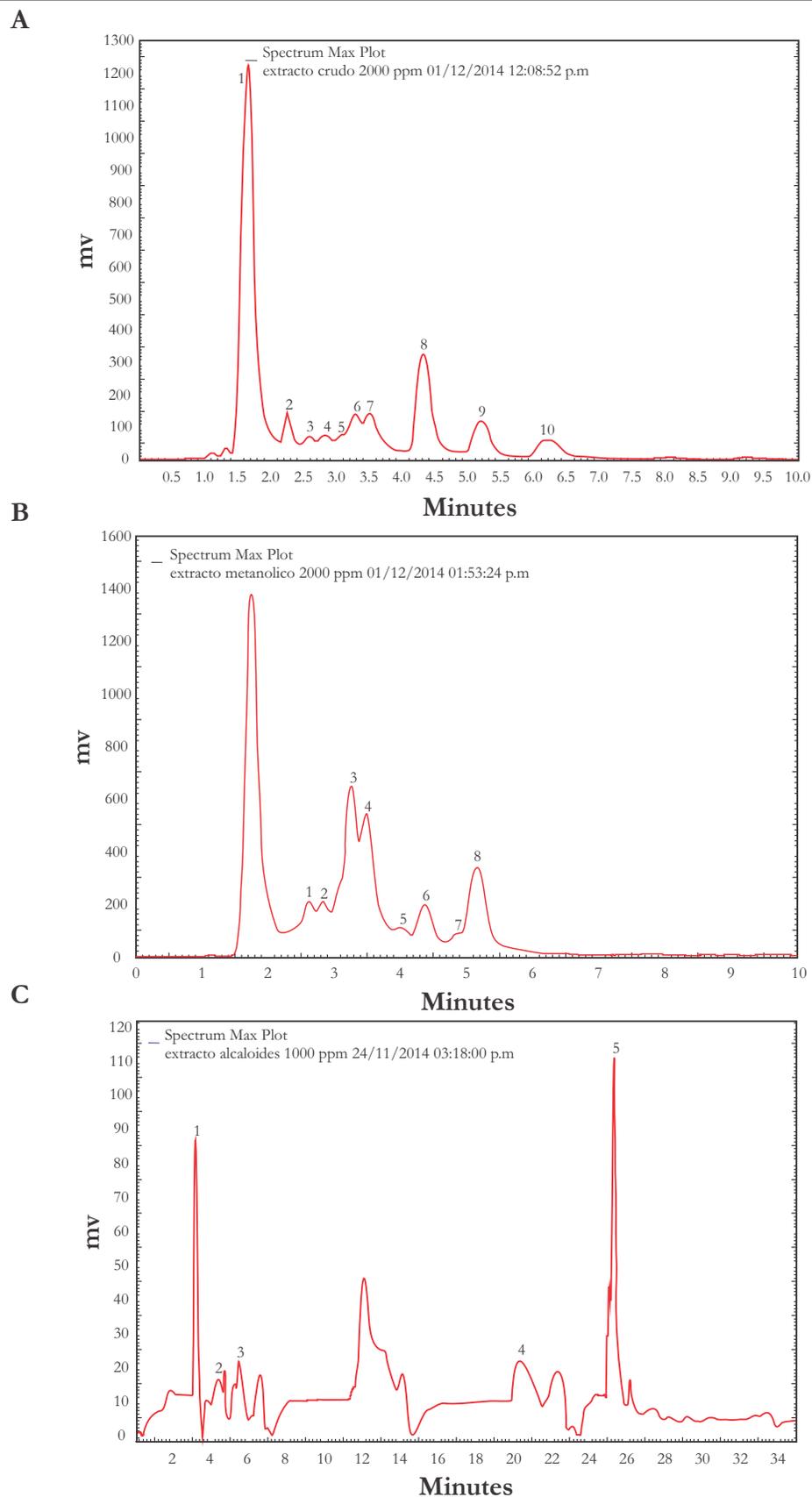


Figure 1. *Rollinia mucosa* degraded seed extract chromatograms. **A.** Ethanol extract, **B.** Acetogenin (F1) fraction, and **C.** Alkaloid (F2) fraction.

The other peaks shown in Fig. 1A. likely correspond to phenolic compounds, which have maximum absorption peaks at 270 nm (Ferrerres *et al.* 2017) and lignans, which have maximum absorption peaks in the 220-225 and 278-281 nm regions (Fuentelba *et al.* 2015).

Fig. 1B. shows the chromatographic profile of the acetogenin (F1) fraction. A total of eight peaks were observed, and with the help of their UV-VIS spectra, it was possible to determine that these correspond to acetogenin compounds.

The alkaloid (F2) fraction's chromatographic profile revealed five peaks (Fig. 1C). With the help of UV-VIS spectra, these peaks were identified as corresponding to isoquinoline alkaloids. According to their chemical nature, these alkaloids were of the aporphynic type, which have maximum absorption peaks at 215, 265, 280, and 294 nm; and of the phenanthrene type, which have maximum absorption peaks at 250 and 279 nm (Hu *et al.* 2010).

Two of the detected metabolite groups in the of the *R. mucosa* seed ethanol extract, namely acetogenin and alkaloids, were expected to have insecticidal activity.

Cytotoxic activity: Median lethal concentrations (LC₅₀) of the raw ethanol extract, the acetogenin fraction (F1), and the alkaloid fraction (F2) on *A. salina* are shown in [Table 1](#).

Insecticidal activity: Increasing concentrations of *R. mucosa* seed extract did not have an overall negative effect on lace bug mortality ([Fig. 2](#)). The highest level of mortality, 86.67 %, was observed after 72 h of treatment with *R. mucosa* seed extract at a concentration of 5 µg/mL. As shown in [Fig. 2](#), the obtained data reveals that a longer the exposure time, leads to increased mortality rates of the lace bug for the five concentrations studied.

The LC₅₀ concentration of *R. mucosa* seed ethanol extract was determined as 74 µg/mL for an exposure time of 72 h. The LC₅₀ concentrations for the other exposure times could not be determined. According to the observed insecticidal effect of the tested concentrations, the most promising concentration range was that between 5 µg/mL and 50 µg/mL; whereas, for a concentration of 100 µg/mL there were no significant rises in insect mortality.

Table 1. Toxicity screening of raw *Rollinia mucosa* seed ethanol extract, its acetogenin (F1) fraction, and its alkaloid (F2) fraction on the brine shrimp, *Artemia salina*.

Repetition	Lethal concentration LC ₅₀ (µg/mL)		
	Ethanol extract	Acetogenin fraction (F1)	Alkaloid fraction (F2)
1	0.171	0.066	0.069
2	0.183	0.120	0.043
3	0.200	0.059	0.036
Average	0.184	0.082	0.0493

The observation that the highest concentrations of seed extract led to lower mortality rates as exposure time increased, prompted us to hypothesize that at low concentrations insects do not detect the extract and easily feed on the impregnated leaves. Thus, mortality increased in the long term. Contrastingly, at high concentrations the extract itself becomes phagorepellent, impeding insect nourishment, and leading to death by starvation.

Interestingly, during the whole experiment female insects were laying eggs; this behavior could be related to the effect produced by the compounds present in the extract (Biddinger *et al.* 2009).

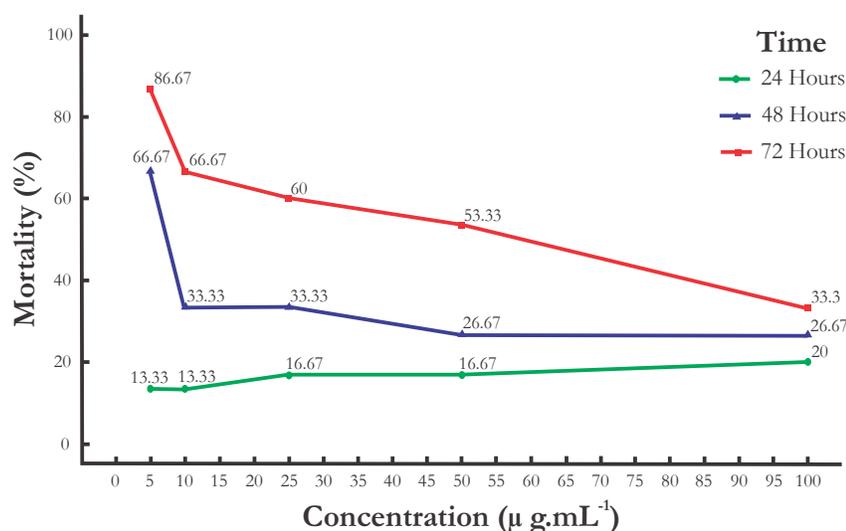


Figure 2. Effect of the concentration of *Rollinia mucosa* seed extract and exposure time on lace bug mortality (lace bug at III, IV, and V nymphal stages were exposed for 24, 48, and 72 hours).

Our results agree with previous reports, in that acetogenins and alkaloids of the Annonaceae family have the same mechanisms of action. It has been reported that the three types of Annonaceae acetogenins of *R. mucosa*, bis-tetrahydrofuran (THF), mono-tetrahydrofuran (THF), and epoxides have cytotoxic potential, as well as pesticidal, herbicidal, and antifeedant activities (Kuo *et al.* 2001). The bioactive potential of these compounds is elicited by means of the reduction of ATP levels. This reduction affects the process of electron chain transport in the mitochondria, leading to cell death (Castillo *et al.* 2010).

There is evidence that isoquinoline alkaloids of to the Annonaceae family have multiple biological activities, including antifungal, cytotoxic, deterrent, repellent, antifeedant, and insecticidal (Torres *et al.* 2007). These compounds inhibit the enzyme acetylcholinesterase, thus impeding the degradation of the neurotransmitter acetylcholine. As the concentration of acetylcholine increases, hyperexcitation of the Central Nervous System (CNS) causes insect death (W02013043031 A1, 2013).

Conclusions

The active compounds of *R. mucosa* extract have great potential as bio controllers of the lace bug (*Corythucha gossypii*), due to their insecticidal and deterrent actions. The results of the present work further support the case for the potential utilization of plant species native to the Colombian Amazon, particularly that of *R. mucosa*, as biocontrol agents. In addition, this study has provided fundamental information about the biological activity reported for the Annonaceae family species. All the above constitutes a starting point for new research proposals.

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Conflict of interests

The authors have no conflict of interests to declare.

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Metabolitos activos de la semilla de *Rollinia mucosa* (Jacq.) Baillon (Annonaceae) como agentes alternativos de biocontrol sobre el insecto fitopatógeno *Corythucha gossypii* (Fabricius).

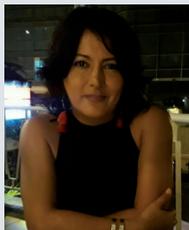
Resumen. El hemíptero, *Corythucha gossypii* (Fabricius) es un insecto que causa daño sustancial en cultivos de más de 24 especies de plantas de las familias Annonaceae, Passifloraceae, Caricaceae, Euphorbiaceae y Solanaceae. En su mayoría estas plantas son de interés económico. Aunque insecticidas comerciales como el Dimetoato (0.1 %) y el Imidacloprid (0.1 %) permiten un manejo eficiente de este insecto-plaga, no se han reportado alternativas botánicas para estos insecticidas sintéticos. En el presente estudio se evaluó el extracto etanólico de la semilla de *Rollinia mucosa* (Jacq.) Baillon, como un biocontrolador de *C. gossypii*. A través de un test de toxicidad con *Artemia salina* se determinó que la concentración del extracto etanólico letal para el 50 % de la población bajo estudio (LC₅₀) fue de 0.184 µg/mL. De igual modo se identificó que las fracciones de acetogeninas (F1) y de alcaloides (F2) de este extracto tienen un LC₅₀ de 0.082 y 0.0493 µg/mL, respectivamente. En el ensayo insecticida con ninfas de *C. gossypii* se observó una mortalidad del 86.67 % después de 72 horas de exposición al extracto etanólico a una concentración de 5 µg/mL. Lo anterior demuestra que el extracto es altamente activo. La caracterización química del extracto evidenció que los principales metabolitos activos que contribuyen a su actividad insecticida son las acetogeninas y los alcaloides.

Palabras clave: *Corythucha gossypii*; *Rollinia mucosa*; Biocontrolador; Acetogeninas; Alcaloides Isoquinolínicos

Metabólitos ativos de *Rollinia mucosa* (Jacq.) Baillon (Annonaceae) como agentes de biocontrole alternativos contra *Corythucha gossypii* (Fabricius): um inseto-praga.

Resumo. *Corythucha gossypii* (Fabricius) é uma praga séria que afeta mais de 24 plantas silvestre e de interesse comercial, pertencentes as famílias Annonaceae, Passifloraceae, Caricaceae, Euphorbiaceae e Solanaceae. Até o momento, inseticidas comerciais como Dimetoato (0.1 %) e Imidacloprid (0.1 %) apresentam um controle eficiente sobre este inseto, entretanto não há reportes de pesticidas de origem vegetal para o seu controle. No presente estudo, o extrato etanólico de *Rollinia mucosa* (Jacq.) Baillon foi avaliado como um controle biológico contra *Corythucha gossypii*. Por meio do ensaio de toxicidade com *Artemia salina* a concentração letal média (LC₅₀) para o extrato etanólico das sementes, suas frações de acetogeninas (F1) e fração de alcaloides (F2) foi de 0.184, 0.082 y 0.0493 µg/mL, respetivamente. Adicionalmente, na avaliação do ensaio inseticida, se obteve uma porcentagem de mortalidade de 86.67 % à concentração de 5 µg/mL após de 72 horas de exposição, demonstrando uma alta atividade do extrato de sementes de *R. mucosa*. Os estudos em relação à caracterização química evidenciaram que os principais metabólitos que aportam à atividade do extrato foram as acetogeninas e alcaloides.

Palavras-chave: Acetogeninas; Biocontrole; Alcaloides Isoquinolínicos; *Corythucha gossypii*; *Rollinia mucosa*

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