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ORIGINAL ARTICLE

Biological activities of *Annona montana* Macfad extracts

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Abstract

Annona montana Macfad is a fruit species of the Annonaceae family. In this study, the phytochemical potential of *A. montana* seeds was investigated. Ethanol and hexane extracts from seeds were evaluated for cytotoxicity and insecticidal activity, phenolic content, and antioxidant capacity. The latter being related to free radical scavenging activity assay (DPPH) and ferric reducing power (FRAP). Exposing *Artemia salina* to both seed extract types reveled their high toxicity with a median lethal concentration (LC50) of $< 10\,\mu g\,mL^{-1}$. Further *A. montana* seed insecticidal activity was evaluated against *Thrips tabaci* L., revealing that the most promising treatments were observed for a concentration of $100\,mg\,L^{-1}$ in both extracts. The ethanol extract resulted in a mortality of 67.5 % and the hexane extract in a 53.3 % mortality. The ethanolic extract of *A. montana* seeds showed the highest total phenolic content: 297.38 mg GAE/100 g of dried extract and 192.66 mg TE/100 g, and 385.46 mg TE/100 g for DPPH and FRAP, respectively. The chemical characterization of both extracts by high performance liquid chromatography (HPLC) revealed the presence of acetogenins. The results obtained indicate that the *A. montana* extracts are a promising source of compounds with insecticidal activity.

Keywords: Annonaceae; antioxidant; cytotoxicity; DPPH, FRAP, insecticidal activity.

1. Introduction

The Annonaceae constitute a family with plesiomorphic traits, in fact it constitutes one the first flowering plant families included in the Ranalean complex. A total of 2400 trees and shrubs species in 130 genera are recognized within the Annonaceae. These are distributed throughout the tropical zones of America, Africa, Indochina, and Malaysia. From a chemical composition perspective, several studies carried out with the Annonaceae reveal that its species possess a wide spectrum of biological activities for pest control [1, 2, 3, 4].

Annona montana, also known as guanábana de monte or maroon soursop, is a tropical plant grown in South America and found in some parts of India. The tree is about 10 m in height and its yellow-green fruit, which is acidic, has several carpels and many light brown seeds and is highly valued for its striking aroma [2]. *A. montana* is used in traditional domestic medicine; infusions of its leaves serve to treat lice, influenza, and insomnia [3, 4].

The toxicity of *A. montana* fruit extracts have been evaluated in cell lines, whereby these were found to inhibit tumor cells growth in colon, breast, prostate, and lung cancers [5, 6]. Several studies have focused on the evaluation of *A. montana* insecticidal activity, namely evaluating seed extracts against insect pests such as Cabbage looper (*Trichoplusia ni*) and the Maize weevil (*Sitophilus zeamais*) in crops grains [7, 8]. *Helicoverpa armigera*, a polyphagous pest that damages the vegetative structures of soybean and corn have been exposed to *A. montana* extracts [9]. Furthermore, *A. montana* leaf and fruit extracts were evaluated against nymphs of *Aphis*

craccivora [10] and *Spodoptera frugiperda* [11]. Furthermore, acetogenin isolates from leaves and twigs have also been assessed against *Oncopeltus fasciatus* [4] and disease vectors, such as *Anopheles gambiae* and *Culex quinquefasciatus* [12] with promising results.

The Annonaceae is the only family with plants producing acetogenins; metabolites that exhibit antitumor, antimalarial, anti-inflammatory, antimicrobial, immunosuppressant, and pesticidal properties. Acetogenins are central in the production of benzylisoquinoline alkaloids [13, 14]. There are no reports of the insecticidal activity of *A. montana* against *Thrips tabaci. T. tabaci* is an important pest found worldwide, infesting more than 300 species of plants and is one of the main plagues of onion crops [15, 16, 17].

Considering the great potential of Annonaceae extracts, we conducted bioprospection assays with *A. montana* seeds. Thus, we evaluated different activities of polar and non-polar seed extracts. Cytotoxicity against *Artemia salina* was first assessed as a potential predictor assay for insecticidal and biological screening for potential bioactive drugs. Next, we studied the insecticidal effect against *T. tabaci* L. and the antioxidant activity value of the extracts, using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) scavenging method and the ferric reducing power method. Further, acetogenin-like compounds were identified and total phenolic content (TPC) of the extracts were determined.

2. Materials and Methods

2.1. *Annona montana* seeds

A. montana seeds were gathered in jurisdiction of Caicedonia, Valle del Cauca, Colombia. The taxonomic identification of the plant took place in the herbarium at Universidad del Quindío with voucher number 38331. In the preparation of the extract, the washing of the seeds with TEGO 51 soap and drying processes at 37 °C. Finally, a mill IKA MF 10 BASIC was used to obtain a fine powder stored under refrigeration [18].

2.2. Extract preparation

To obtain the polar extract of *A. montana* seeds, passive maceration was carried out at room temperature. The sample was subjected to regular agitation for one week using ethanol as the solvent, in a sample-solvent ratio of 1:4 (w/v) and filtration under vacuum. The non-polar extract was obtained using the Soxhlet extraction technique employing hexane as the solvent, in a sample to solvent ratio of 1:5 (v/w), for 24 hours [18]. The solvent was rotary evaporated at low pressure, concentrated with nitrogen gas, and kept at 4 °C.

2.3. Cytotoxic activities against *Artemia salina*

The cytotoxic activity analysis was made with the methodology described by McLaughlin [19, 20] with some modifications. *Artemia salina* eggs were hatched in a 3.7 % artificial seawater solution, and then incubated at 30 °C for 48 hours. *A. montana* seed extracts of different concentrations (0.5 mg L^{-1}, 1 mg L^{-1}, 3 mg L^{-1}, 5 mg L^{-1} and 10 mg L^{-1}) were then evaluated, adding 10 *A. salina* nauplii to each experimental unit. Two controls were prepared; the first with artificial seawater solution, and the second with ethanol solution. All tests were performed three times. After 24 hours the numbers of alive and motionless *A. salina* larvae were counted.

2.4. Evaluation of the insecticidal activity against *Thrips tabaci*

Morphological identification of *T. tabaci*: Thrips larvae were taken from onion crops in vereda La Florida, municipality of Pereira, Risaralda, Colombia. Infested leaves were plucked, stored hermetically in plastic bags, and carried to laboratory for analysis. In the laboratory, larvae were separated from the leaves and placed in a Petri box containing a solution of ethanol (70 %) [21]. Larvae were identified with the help of a microscope, following the taxonomic keys proposed in previous studies [22].

2.5. Median lethal concentration (CL50) assessment

The biocidal activity of the extract was evaluated in the farm where thrips larvae were sampled. Bioassays were conducted at room temperature ((21 ± 1) °C); at a relative humidity (RH) of (50 ± 10) %. *T. tabaci* larvae were net-collected from onion crops, following the methodology implemented in previous studies [23]. A randomized experimental design with eight treatments and six repetitions was followed. The extracts were diluted with dimethyl sulfoxide (DMSO) and distilled water to obtain different concentrations (10 mg L^{-1}, 50 mg L^{-1} and 100 mg L^{-1}). These doses were tested in independent trials and with the same review and control treatment. Two controls were employed, a blank containing the same amount of dimethyl sulfoxide (DMSO) was used to dissolve the extracts and Lorsban 4EC insecticide, which belongs to the chlorpyrifos family.

2.6. Determination of total phenolic content (TPC)

A. montana extracts were determined following the Folin Ciocalteu method, as previously reported [24]. Briefly, 50 µL of the extract (12 500 mg L^{-1}) were mixed with 120 µL of water with 50 µL of the Folin–Ciocalteu reagent were dissolved in water (1:20) and 80 µL of KOH (0.175 M). Absorbance was measured at 760 nm. A calibration curve was plotted using gallic acid as the reference standard with a concentration range of 5 mg L^{-1} to 80 mg L^{-1}. The results were expressed in milligrams of gallic acid equivalents per 100 g of sample (mg GAE/100 g of extract).

2.7. DPPH free radical scavenging activity assay

DPPH was performed following a method previously described [25]. Briefly, 10 µL of the extract (25 000 mg L^{-1}) were mixed with 200 µL of DPPH–ethanol solution at 50.7 µM (20 mg L^{-1}). Absorbance was measured at 517 nm, and a calibration curve was plotted using Trolox as the reference standard with a concentration range of 10 µM to 400 µM. Results were expressed in milligrams of Trolox equivalents per 100 g of extract (mg TE/100 g extract).

2.8. Ferric reducing antioxidant power FRAP

This analysis was performed according to a previously proposed method [26]. FRAP reagent was prepared mixing a 300 mM acetate buffer solution (pH 3.6) with 10 mM TPTZ, 40 mM HCl, and 20 mM FeCl3 solution (ratio, 10:1:1). Subsequently, 150 µL of the FRAP reagent were added to 20 µL of extract (12 500 mg L^{-1}). The reaction mixture was incubated at 37 °C for 30 min, and the absorbance was measured at 593 nm. A calibration curve was plotted using Trolox as the reference standard with a concentration range of 20 mg L^{-1} to 100 mg L^{-1}. Results were expressed in milligrams of Trolox equivalents per 100 g of extract (mg TE/100 g extract).

2.9. Chemical characterization

For the analysis of the acetogenins, *A. montana* crude extracts were diluted in a chloroform – water solution in a 1:1 ratio and a liquid-liquid extraction was carried out following the methodology described elsewhere [27].

High performance liquid chromatography (HPLC) analysis: This analysis was made in a Jasco 2000 plus chromatograph, equipped with (i) a quaternary gradient pump (PU-2089 Plus), (ii) an intelligent autosampler (AS-2059Plus), (iii) a column oven (CO-2065 Plus), and (iv) an intelligent diode array detector (MD-2015 Plus). For fraction analysis, an ODS2 Spherisil reverse phase column (250 mm × 4.6 mm i.d, 5.0 µm) and a Spherisil pre-column (5.0 mm × 4.6 mm i.d, 5.0 µm) were used (CAPITAL HPLC, Broxburn, United Kingdom). An isocratic method, with a mobile phase of water (A) and acetonitrile (B) in a 30:70 ratio, at a rate of 1 mL min^{-1} with a running time of 50 min, was employed. A volume of 20 µL of sample at 100 µg mL^{-1} was injected. Acetogenins were identified using the UV-Vis spectrum of the Bullatacina standard [18].

3. Data analysis

Cytotoxic and biocidal activity results were expressed as mean ± SD. An analysis of variance (ANOVA) was used to test for the difference of means; Tukey's test, at a 5 % significance level, was employed for the toxicity test with Artemia, and a Hoteling test with Bonferroni corrected level of $P < 0.05$ was used for insecticidal activity. Mean lethal concentration (LC50) was determined using Probi analysis. Results were obtained with the help of the statistical software Infostat version 2008.

4. Results

Cytotoxic activity against *A. salina*: **Table 1** shows cytotoxicity results, all of the *A. montana* seed extract doses exerted larvicidal activity on *A. salina*, revealing statistical difference between treatments and controls. The dose of 10 mg L^{-1} led to the highest percentage of *A. salina* mortality (> 80 %). As revealed by *A. salina* experiments, the mean lethal concentration (LC50), calculated after 24 h, for the non-polar extract was 3.58 mg L^{-1} and for the polar extract was 3.22 mg L^{-1}. This supports previous findings that both extract types were highly active [23, 27].

4.1. Insecticide activity against thrips

Morphological identification of the thrips from onion crops revealed that the insects belong to the species *T. tabaci*, diagnostic features are shown in **Figure 1**, and entail: three thoracic segments (Figure 1A), three pairs of thoracic legs (Figure 1B), ten abdominal segments (Figure 1C), and one antenna divided in seven sectors (Figure 1D); there were no projecting parts. This description agrees with that reported in the literature [28].

In the test against *T. tabaci* larvae, all doses of the polar and non-polar *A. montana* seed extracts had a biocidal effect on the larvae (**Figure 2**). The level of response depended on extract concentration. No significant differences were noted between the treatments with polar and non-polar extracts, but there were significant differences between extracts and control.

The highest percentage of mortality was reached working at an extract (polar and non-polar) concentration of 100 mg L^{-1}; at 24 hours, the polar extract led to a mortality of 67.5 % and the non-polar extract to a mortality of 53.3 %. The control did not exert mortality and the Lorsban

Table 1. Mortality of *Artemia salina* larvae upon 24-h exposure to different concentrations of (A) polar extract and (B) non-polar extracts of *Annona montana* seeds. Values followed by different letters indicate differences between treatments according to a Tukey test at a 5 % probability level.

Extracts	Concentration ($mg\,L^{-1}$)	Mortality (%)	Median lethal concentration (LC50)
Ethanol	0.5	34.7 ± 7.5^a	$3.2\,mg\,L^{-1}$
	1	56.45 ± 9.12^b	
	3	82.30 ± 3.25^b	
	5	86.10 ± 2.40^b	
	10	94.40 ± 7.92^b	
Hexane	0.5	22.30 ± 0.71^a	$3.58\,mg\,L^{-1}$
	1	34.45 ± 4.88^a	
	3	37.00 ± 8.49^a	
	5	66.10 ± 2.26^b	
	10	84.75 ± 4.60^b	
Negative control	–	100^b	–
Positive control	–	0^c	–

4EC insecticide control led to a percentage of mortality (78 %) at a concentration of $96\,mg\,L^{-1}$. The median lethal concentration (LC50) for the polar extract was $68\,mg\,L^{-1}$, and for the hexane extract it was $97.5\,mg\,L^{-1}$. These results reaffirm the fact that the highest lethality, found in the present study, corresponds to the ethanolic extract. Thus, these values show that the extracts of *A. montana* can control *T. tabaci.*

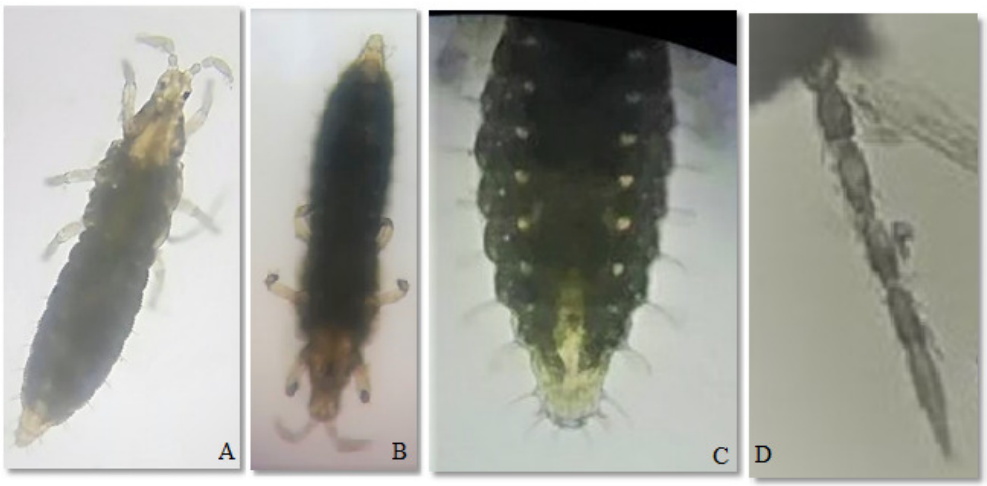

Figure 1. External characters of *Thrips tabaci* in its larval stage II (A) Body, (B) Legs, (C) Abdominal segments, and (D) Antenna.

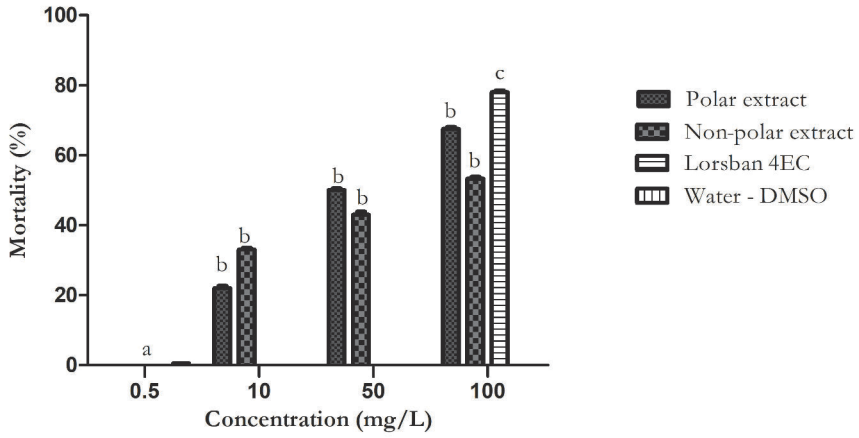

Figure 2. Mortality of *Thrips tabaci* larvae exposed to different concentrations of (A) polar extract (B) and non-polar extract of *Annona montana* seeds for 24 h. Bars sharing a letter did not differ significantly according to a Tukey test at a 5 % probability level.

4.2. Total phenolic content and antioxidant activity

Table 2 shows the results of TPC determined using the Folin-Ciocalteu method and antioxidant capacity by the FRAP and DPPH methods. The extracts revealed TPC mean values between 220 mg to 300 mg GAE/100 g of extract, agreeing with values observed by other authors [29]. The ethanol extract showed the highest total phenolic content. As per T-test, no significant differences were noted in the TPCs. The extracts showed antioxidant activity in a range of 150 mg TE/100 g to 400 mg TE/100 g of extract. The ethanol extract had the highest antioxidant activity for both methods. However, using the FRAP method, extracts showed increased antioxidant activity. Based on the results of ANOVA at a significance level of 5 %, there were significant differences between the antioxidant activities of extracts. Furthermore, TPC revealed linear correlations between antioxidant activity assessed and method: FRAP ($r = 0.62$) and DPPH ($r = 0.57$).

4.3. Acetogenin characterization

A total of 25 acetogenin-like compounds were recognized (**Figure 3**) via high resolution liquid chromatography (HPLC) on seed extracts, based on their UV-Vis spectra with absorbance maxima between 200 nm and 220 nm [27]. Of the 25 compounds, 12 were from the polar extract, while the remaining 13 were from the non-polar extract. This could be indicative of the presence of a wide range of acetogenin compounds. These findings may help explain their difference in biological

Table 2. Total phenolic content and antioxidant activity as assessed using the DPPH and FRAP methods of the ethanolic and hexane extracts of *Annona montana* seeds.

Extracts	**Total phenolic content** (mg GAE/100 g of extract)	**FRAP method** (mg TE/100 g of extract)	**DPPH method** (mg TE/100 g of extract)
Ethanol	297.38 ± 16.23^{b}	385.46 ± 13.57^{c}	192.66 ± 27.52^{a}
Hexane	220.63 ± 20.74^{b}	360.05 ± 16.15^{c}	155.49 ± 31.75^{a}

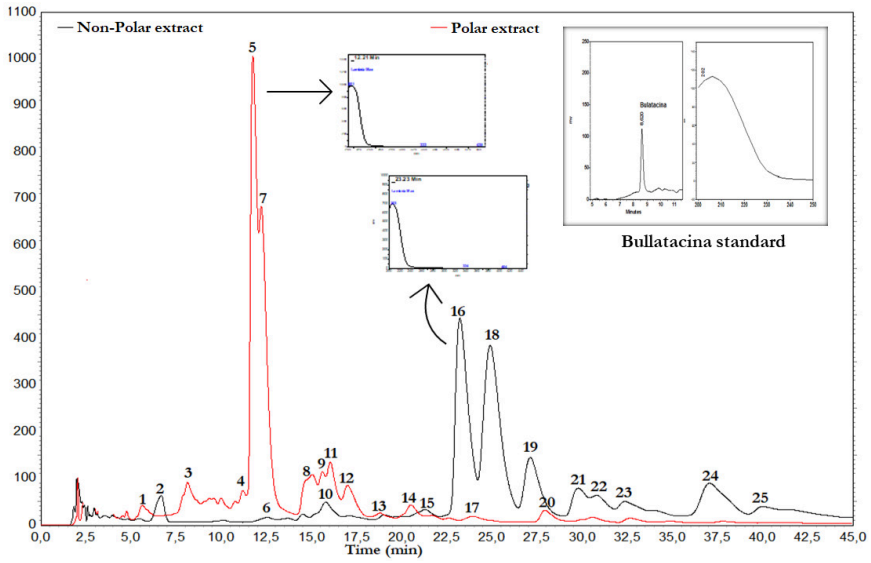

Figure 3. Chromatographic profile of acetogenins compounds check-up to polar and non-polar extracts of *Annona montana seeds.*

activity; it has been reported that the structural characteristics of an acetgonin determine its type of bioactivity as well as its degree of lethality [30–32]. The difference in the composition could explain the higher insecticidal activity of the polar extract.

5. Discussion

Acetogenins are one of the main substances present in seeds of the Annonaceae family [13, 14]. According to previous studies, *A. montana* seeds contain different types of acetogenins with toxic, insecticidal, and feeding dissuasive effects [4, 10]. Reported acerogenins include, squamocin and molvizarin (bis-tetrahydrofuran acetogenins); montalicins A-E; cis-annoreticuins, montalicins F, I, and J, that are mono-tetrahydrofuran [4, 30, 33]; linear acetogenins [31]; and acetogenins with a tetrahydropyran ring [34].

Insecticidal activity was observed after 24 h of treatment, thrips larvae were motionless and had a yellowish color; these effects can be related acetogenin compounds present in the studied seed extracts. These compounds inhibited the mitochondrial complex I (NADH: ubiquinone oxidoreductase), either by contact or ingestion, blocking sodium channels and inducing paralysis followed by death [35]. It has also been reported that other acetogenins present in *A. montana* have insecticidal and deterrent effects [30]. Furthermore, the variability observed in acetogenin modes of action may depend on the different functional groups present in these metabolites [36].

Other important types of compounds reported in *A. montana* seeds correspond to isoquinoline alkaloids; these compounds affect acetylcholinesterase or sodium channels [37]. Some phenolic compounds are toxic to mitochondria because of their broad-spectrum electron transport chain inhibition. Terpenoids are another type of compounds with insecticidal activity, their effects on insects range from repellency, exerted as a deterrent to feeding and oviposition, to interference with growth and development and, ultimately, acute toxicity [38].

When assessing seed extract antioxidant activity, two methods were implemented. This was necessary because tow response mechanisms were known to involved, and it was impossible to determine their activity using a single method. The FRAP method measures the ability of the compounds to reduce Fe III to Fe II, forming a blue complex with 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ). Whereas the DPPH method involves the reduction of the DPPH radical, which provides a rate to estimate the ability of a compound to capture radicals [39].

There are no previous reports of antioxidant activity in *A. montana* seed extracts; however, extracts of *A. montana* fruit flesh, with IC50 less than 100 ppm, have been characterized as rich in antioxidants [40]. TPC was not found to have a strong linear correlation with the antioxidant activity assessed using both methods. This is possibly due to antioxidant activity mostly attributed to phenolic-type compounds [41, 42]. These compounds have been reported in fruit flesh and leaves of other species within the Annonaceae [41, 43, 44].

Finally, Ethanolic and Hexane extracts of *A. montana* could make a real contribution to the task of controlling thrips populations; it is necessary to continue the field assays to establish their degree of incidence. This considers that in different countries biodegradable products, botanical insecticides, and biopesticides have been successfully used to get reductions of the pest population of the order of 58.14 % [17, 45, 46] and chemical characterization studies should be expanded and carried out to allow greater safety of the compounds present.

6. Conclusion

In this work, we have expanded the bioprospecting of *A. montana* seed polar and non-polar extracts by studying their cytotoxic, insecticidal and antioxidant activities. When Thrips were exposed to these extracts, mortality rates were high at low extract concentrations. Thus, *A. montana* seed extracts have great potential as bio insect pest controllers. Although acetogenins and phenolic compounds were identified within the extracts, their chemical characterization should be conducted.

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8. Conflict of interest

The authors declare that there are no conflicts of interest.

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Actividades biológicas de extractos de *Annona montana* Macfad

Resumen: *Annona montana* Macfad es una especie frutal de la familia Annonaceae. En este estudio se investigó el potencial fitoquímico de las semillas de *A. montana*. Se evaluaron los extractos etanólico y hexánico de las semillas para determinar citotoxicidad y actividad insecticida, contenido fenólico y capacidad antioxidante; esta última, relacionada con los ensayos de actividad de captación de radicales libres (DPPH) y de poder reductor férrico (FRAP). La exposición de *Artemia salina* a ambos tipos de extractos de semillas reveló su alta toxicidad, con una concentración letal media (LC50) de $< 10\,\mu g\,mL^{-1}$. La evaluación posterior de la actividad insecticida de *A. montana* contra *Thrips tabaci* L. reveló que los tratamientos más promisorios se observaron en la concentración de $100\,mg\,L^{-1}$ en ambos extractos. El extracto etanólico resultó en una mortalidad de 67.5 % y el hexánico, en una mortalidad de 53.3 %. El extracto etanólico de semillas de *A. montana* mostró el contenido fenólico más alto: 297.38 mg GAE/100 g de extracto seco, y 192.66 mg TE/100 g y 385.46 mg TE/100 g para DPPH y FRAP, respectivamente. La caracterización química de ambos extractos por cromatografía líquida de alta resolución (HPLC) reveló la presencia de acetogeninas. Los resultados indican que los extractos de *A. montana* son una fuente promisoria de compuestos con actividad insecticida.

Palabras Clave: Annonaceae; antioxidante; citotoxicidad; DPPH, FRAP, actividad insecticida.

Atividades biológicas de extratos de *Annona montana* Macfad

Resumo: *Annona montana* Macfad é uma espécie de fruta da família Annonaceae. Neste estudo, pesquisamos o potencial fitoquímico de sementes de *A. montana*. Estratos de sementes em etanol e hexano foram avaliados quanto a sua citotoxicidade, atividade inseticida, conteúdo fenólico e capacidade antioxidante. Este último relacionado a um ensaio de atividade de eliminação de radicais livres (DPPH) e poder redutor férrico (FRAP). Expor *Artemia salina* aos dois tipos de estratos de semente revelou sua alta toxicidade com uma concentração letal média (LC50) de $< 10\,\mu g\,mL^{-1}$. Adicionalmente, a atividade inseticida das sementes de *A. montana* foi avaliada contra *Thrips tabaci* L. Os tratamentos mais promissórios foram observados a uma concentração de $100\,mg\,L^{-1}$ nos dois extratos. O estrato em etanol resultou numa mortalidade de 67.5 % e os estrato em hexano resultou em 53.3 % de mortalidade. O estrato etanólico de semente de *A. montana* apresentou o maior conteúdo fenólico total: 297.38 mg GAE/100 g de extrato seco e 192.66 mg TE/100 g e 385.46 mg TE/100 g para o DPPH e FRAP, respectivamente. A caracterização química dos dois extratos por cromatografia líquida de alta resolução (HPLC) revelou a presencia de acetogeninas. Os resultados obtidos indicam que os extratos de *A. montana* são uma fonte promissória de compostos com atividade inseticida.

Palavras-chave: Annonaceae; antioxidante; citotoxicidade; DPPH, FRAP, atividade inseticida

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