TOXICITIES OF *Annona* DERIVATIVES AND SEMI-PURIFIED FRACTIONS AGAINST *Zabrotes subfasciatus* †

[TOXICIDAD DE LOS DERIVADOS DE *Annona* Y FRACCIONES SEMI-PURIFICADAS CONTRA *Zabrotes subfasciatus*]

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SUMMARY

Background. The Annonaceae botanical family is a promising source of new insecticidal molecules that can be used to protect stored beans against Bruchinae beetles due to its variety of bioactive compounds such as alkaloids and acetogenins. Bruchinae beetles are major pests of stored beans in the world. These beetles feed on stored beans and they promote high levels of damages. **Objective.** The present study assessed the lethal and sublethal effects of crude ethanolic extracts prepared from different parts (leaves, branches and seeds) of *Annona montana, Annona mucosa, Annona muricata and Annona reticulata* against the Mexican bean weevil, *Zabrotes subfasciatus* (Boehman). **Methodology.** It was performed toxicological bioassays by spraying ethanolic extracts and fractions from *Annona* species on grains surface. The treated grains were infested with adults of *Z. subfasciatus*. It was evaluated the number of dead insects, eggs, F_1 progeny and damaged grains. Ethanolic extracts obtained by cold maceration [ratio 1:5 (v p^{-1})] were applied on grains surface (cv. Bolinha) at 1,500 ppm [extract (mg) grains $(kg)^{-1}$]. **Results.** The seed extract from *A. mucosa* promoted 100% of mortality of *Z. subfasciatus* and completely inhibited the oviposition, the F_1 progeny emergence and the damage on grains. In addition, it was also estimated the LC_{50} of the ethanolic extracts from seeds of *Annona* species. Interestingly, lethal concentrations varied according to *Z. subfasciatus* sex, and females supported higher doses than males. The ethanolic extract from seeds of *A. mucosa* presented the lowest LC_{50} value (571.82 mg kg^{-1}) among all extracts. Thereby, it was submitted to liquid-liquid partition producing a hexane fraction and a remaining hydro-methanol phase. The former fraction killed 100% of insects whereas the later killed 20% of insects, but both of them promoted sublethal effects on *Z. subfasciatus* reducing the number of eggs and F_1 progeny. **Implication.** The ethanolic extracts of *A. mucosa*, *A. montana*, *A. muricata* and *A. reticulata* can be used as raw material for the formulation of a botanical insecticide that can help small farmers. **Conclusion.** The ethanolic extracts from seeds of *A. mucosa*, *A. montana*, *A. muricata* and *A. reticulata* are highly toxic to *Zabrotes subfasciatus* and protected the stored beans.
Keywords: Annonaceae; botanical pesticides; Mexican bean weevil; lethal concentration.

RESUMEN

Antecedentes. Las plantas de Annonaceae son una fuente prometedora de nuevos compuestos insecticidas, tales como alcaloides y acetogeninas, para el control de Brúquidos quienes dañan granos almacenados. Los Brúquidos son las principales plagas de frijoles almacenados en el mundo. Los Brúquidos se alimentan de frijoles almacenados y promueven altos niveles de daños. **Objetivo.** Se estudió el efecto tóxico de los extractos etanólicos preparados a partir de diferentes partes (hojas, tallos y semillas) de *Annona montana, Annona mucosa, Annona muricata* y *Annona reticulata* sobre el gorgojo pinto del frijol, *Zabrotes subfasciatus* (Boheman) (Coleoptera: Chrysomelidae: Bruchinae). **Metodología.** Se realizaron bioensayos toxicológicos mediante pulverización de extractos etanólicos y fracciones de especies de *Annona* sobre los granos. Los granos tratados fueron infestados con los adultos de *Z. subfasciatus*. Se evaluó el número de insectos muertos, huevos, progenie F_1 y granos dañados. Extractos etanólicos, obtenidos por la técnica de maceración en frío [proporción 5:1 (v m^{-1})], se aplicaron en la superficie de los granos (frijoles cv. Bolinha) 1,500 ppm [extracto (mg) grano $(Kg)^{-1}$]. **Resultados.** El extracto de las semillas de *A. mucosa* promovió el 100% de mortalidad de *Z. subfasciatus* e inhibió la oviposición, la progenie F_1 y el daño a los granos. Además, se estimó el CL_{50} de los extractos etanólicos de la semilla de *Annona*. Las concentraciones letales varían de acuerdo con el sexo de *Z. subfasciatus*, las hembras soportaron una dosis mayor que los machos. El extracto etanólico de semillas de *A. mucosa* presentó la CL_{50} más baja entre todos los extractos. Por lo tanto, el extracto fue fraccionado en dos, una fracción de hexano y una fase hidro-metanólica. La fracción hexano mató al 100% de los insectos mientras la fracción fase hidro-metanólica mató al 20% de los insectos, sin

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embargo, ambos redujeron el número de huevecillos, la viabilidad para huevecillos, progenie y el daño en los granos. **Implicaciones.** Los extractos etanólicos *A. mucosa*, *A. montana*, *A. muricata* y *A. reticulata* pueden usarse como materia prima para la formulación de un insecticida botánico que puede ayudar a los pequeños agricultores. **Conclusión.** Los extractos etanólicos de la semilla de *A. mucosa*, *A. montana*, *A. muricata* y *A. reticulata* son altamente tóxicos para *Z. subfasciatus* y protegieran los frijoles almacenados.
Palabras clave: Annonaceae; plaguicidas botánicos; gorgojo pinto del frijol; concentración letal.

INTRODUCTION

For centuries, inorganic insecticides (e.g. inorganic sulfur) and insecticidal plants have played an important role in pest control (Oberemok *et al.*, 2015), what can now be recovered for farm systems aiming sustainability. During the green revolution, synthetic pesticides replaced and displaced botanical ones to an inexpressive position in pest control of major agricultural crops (Isman, 2006). However, there is an increasing interest for more sustainable farming systems depending less on synthetic pesticides due to the negative effects they promote on the environment and human health (Campos *et al*., 2018). Inevitably, it forces a different pest management approach that relies on alternative methods to synthetic pesticides. Some ways to achieve such goal is to elaborate plant-based insecticides that can be produced by farmers or to reutilize residues from an industrial process to formulate economically accessible botanical pesticides for farmers. In this approach, efficient and sustainable separation of natural products from agro-industrial wastes constitutes an opportunity to change problems into ecofriendly solutions to agriculture (Zuin and Ramin, 2018).

In Brazil, some *Annona* species can be suitable sources of insecticidal compounds to protect stored grains against insect-pests (Ribeiro *et al*., 2013; Gonçalves *et al*., 2015; Ribeiro *et al*., 2016). The seeds from *Annona muricata* L. and *Annona atemoya* Mabb. (*A. cherimolia* x *A. squamosa*), for example, are an industrial residue that could be incorporated in botanical insecticides due to the presence of insecticidal acetogenins that are efficient against many insect-pests (Seffrin *et al*., 2010; Moghadamtousi *et al*., 2015). Annonaceae acetogenins (ACGs) are a class of compounds composed of long chain fatty acids (C-32/C-34) linked with a 2-propanol unit and a terminal saturated or unsaturated subunit γ-lactone (Alali *et al*., 1999). They have oxygenated functional groups such as hydroxyls, ketones, epoxides, and tetrahydrofuran and tetrahydropyran rings (Li *et al*., 2008). The insecticidal properties of acetogenins are compared to rotenone, which is a potent inhibitor of mitochondrial complex I (Esposti *et al*., 1994; Zafra-Polo *et al*., 1996). To date, the insecticidal activity of 42 species from Annonaceae family has been verified on 65 insect species from different insect orders (Krinsk *et al*., 2014). According to literature, the genera of Annonaceae that present species with insecticidal activity on stored grain pests are *Annona*, *Xylopia*, *Duguetia*, *Dennettia* e *Monodora* (Krinsk *et al*., 2014).

The genus *Annona* presents around 200 species in the tropical region of South America, Central America and Africa (Maas, 2009), occurring in Brazil 83 species distributed by the biomes: Cerrado, Amazon Forest, Caatinga, Atlantic Forest and Pantanal (Maas *et al*., 2003). Many of these Annonaceae species produce edible fruits that can be used by food industry and generate seed residues. For the present study, *Annona montana* Macfad., *Annona mucosa* Jacq., *A. muricata* and *Annona reticulata* Linnaeus were selected due to their edible fruits, wide distribution in Brazil and possibility of cultivation. In the present study, the lethal and sublethal effects of *Annona* ethanolic extracts prepared from different plant parts were evaluated against *Zabrotes subfasciatus* (Boheman) (Coleoptera: Chrysomelidae: Bruchinae), a major pest of stored beans (*Phaseolus vulgaris*). The Mexican bean weevil and other Bruchinae beetles promote high levels of damages on stored beans in tropical areas and the Mediterranean region (Abate and Ampofo, 1996; Tuda, 2007). Therefore, it is very important to develop efficient and accessible methods to control them at warehouses.

MATERIAL AND METHODS

Obtainment of plant material and extract preparation procedures

The details regarding *Annona* species collecting and identification is presented in Table 1. The leaves, seeds and branches of *Annona* species were separately dried at 40ºC for 72 hours and then they were powdered in a knife mill. The *Annona* powders were submitted to a chemical extraction using organic solvent ethanol (analytical grade, 99.5%) at a ratio of 5:1 (v m^{-1}) (ethanol: *Annona* powder). These *Annona* powders were mixed with ethanol during 10 minutes and rested for 72 hours for three consecutive times. The solutions were filtered with filter paper (80 g m^{-2} and porosity of 3 μm) and ethanol was evaporated with a rotary evaporator at 50°C, -600 mmHg and 65 RPM. The ethanolic extracts obtained and their respective yields in brackets were: *A. montana* [leaves (11.79%), branches (3.59%) and seeds (20.23%)], *A. mucosa* [leaves (9.99%), branches (0.99%) and seeds (18.79%)], *A. muricata* [leaves (14.09%), branches (6.04%) and seeds (21.1%)] and *A. reticulata* [leaves (9.92%), branches (3.33%) and seeds (27.85%)].

Table 1 - Annonaceae species used in the study: harvesting data.

Species	Plant parts	Collection site	Date of collection	Voucher number[1]
Annona montana Macfadyen	Leaves, branches and seeds	Campus ESALQ/USP, Piracicaba, SP (22º42'28,2" S; 47º37'59.4" O; altitude: 537 m)	March 21st, 2011	121203
Annona mucosa Jacquin	Leaves, branches and seeds	Campus ESALQ/USP, Piracicaba, SP (22º42'28,5" S; 47º37'59.6" O; altitude: 534 m)	March 17th, 2011	120985
Annona muricata Linnaeus	Leaves, branches and seeds	Campus ESALQ/USP, Piracicaba, SP (22º42'25,4" S; 47º37'43,9" O; altitude: 576 m)	April 12nd, 2011	123317
Annona reticulata Linnaeus	Leaves, branches and seeds	Farm São Luís, Descalvado, SP (21º52'58,0" S; 47º40'38,0" O; altitude: 679 m)	April 2nd, 2011	123318

[1] Specimens were deposited at the ESA Herbarium of the Department of Biological Sciences, ESALQ/USP, in Piracicaba, SP.

Fractionation of the ethanolic extract from seeds of *A. mucosa*.

A liquid-liquid fractionation in a separation funnel with organic solvents of different polarities was performed to separate the chemical compounds in the ethanolic extract from seeds of *A. mucosa*, which was the most promising treatment. The crude extract (105 g) was solubilized in 525 mL of methanol:water 8:2 (v/v) and 525 mL of hexane was added to the funnel in order to produce two immiscible phases. The funnel was mixed and then rested until two different phases were formed, the hydro-methanol phase with 27.81% of the total mass and the hexane one with 63.71%. The organic solvents were evaporated using the same procedures described above.

Bioassays with *Annona* extracts and semipurified fractions

Sample units consisted of Petri dishes (6.5 cm diameter × 2 cm high) with 10 g of bean grains cv. Bolinha treated with *Annona* ethanolic extract (1,500 mg kg^{-1}) or semipurified fraction (581.58 mg kg^{-1}). Sample units were infested with ten adults (5 male + 5 female) of *Z. subfasciatus* aging 0-24 hours. Experimental design for all bioassays was completely randomized with 10 replications per treatment.

After five days, the number of dead adults and eggs on grains surface were counted, and after 56 days it was counted the number of adults emerged from grains and the total amount of damaged bean grains. Insects were considered dead when they did not respond to the touch of brush after one minute; and they were retrieved from sample units when dead. *Annona* extracts and fractions solubilized in acetone:methanol (1:1) were applied on 100 g of grains inside plastic bags (2 L) using a microatomizer gun connected to a pneumatic pump regulated to provide a spray pressure of 0.5 kgf cm^{-2} and volume of 30 L t^{-1} (3 mL 100 g^{-1}). After two hours inside an airflow chamber, dried bean samples (10 g) were infested with five couples of *Z. subfasciatus.* Adults of *Z. subfasciatus* used in bioassays came from a laboratory colony conducted under controlled conditions (25 ± 2°C, 60 ± 10 % R. H. and of 14 light: 10 dark hours) inside glass canisters with *Phaseolus vulgaris* grains cv. Bolinha.

Estimations of concentration-response curves of *Annona* seed extracts

In order to estimate the LC_{50} and LC_{90} of ethanolic extracts from seeds of *A. montana*, *A. mucosa*, *A. muricata* and *A. reticulata* (most active treatments), the same bioassay procedures described previously were adopted (item 2.3). However, the reference concentrations [a range from 100 to 2,500 mg kg^{-1} (mg of extract per kg of grains)] used to estimate them were calculated using the formula proposed by Finney (1971) based on preliminary bioassays.

Data analysis

Lethal concentrations (CL_{50} and CL_{90}) of ethanolic extracts from *Annona* seeds were estimated using a binomial model with log-log complement function (gompit model) with the Probit Procedure of SAS software version 9.2. For all the other data from bioassays with *Annona* ethanolic extracts and semipurified fractions it was used software "R"(version 2.15.1) adopting Generalized Linear Models (GLM) (Nelder & Wedderbum, 1972) with medium-normal probability simulation envelope graph to verify the model´s fit quality (Demétrio and Hinde, 1997; Hinde and Demétrio, 1998). In the instance of significant differences between treatments (ethanolic extracts and semipurified fractions), multiple comparisons tests (Tukey's *post hoc* test, $p < 0.05$) were executed using the glht function of the multicomp package.

Table 2. Lethal and sublethal effects (means ± SE) of ethanolic extracts from *Annona* species (1,500 mg kg^{-1} (mg of extract per kg of grains)) against *Zabrotes subfasciatus*.

Plant structure	Mortality[1]	nº eggs/ sample[1]	F_1 Progeny[1]			Viability (%) (egg-adult) [2]	Sex ratio[3]	Damaged grains (%) [2]
			Males	Females	Total			
Annona montana								
Leaves	5.0±2.2	18.6±4.8 b	8.9±1.8 b	10.3±10.9 b	1.2±3.8 b	82.8±9.1 ab	0.52±0.04	38.3±5.8 b
Branches	1.0±1.0	22.5±4.3 b	6.9±1.8 b	6.9±1.7 b	13.8±13.3 b	53.0±12.4 b	0.49±0.08	31.1±7.4 b
Seeds	100.0±0.0[4]	0.5±0.5 c	0.2±0.2 c	0.2±0.2 c	0.4±0.4 c	--	--	0.3±0.3 c
Control	2.0±1.3	82.5±3.5 a	37.6±2.5 a	34.0±1.7 a	71.6±3.5 a	86.7±1.4 a	0.48±0.01	95.2±1.3 a
F	1.65	61.01	65.92	60.34	68.61	6.19	0.72	68.36
P value	0.212 [ns]	<0.0001	<0.0001	<0.0001	<0,0001	0.0061	0.4532 [ns]	<0.0001
Annona mucosa								
Leaves	14.0±4.0 a	1.8±1.3 b	0.3±0.3 b	0.7±0.6 b	1.0±0.9 b	--	--	2.1±1.7 b
Branches	0.0±0.0[4]	2.7±1.3 b	1.0±0.5 b	1.0±0.7 b	2.0±1.2 b	--	--	3.7±2.3 b
Seeds	100.0±0.0[4]	0.0±0.0[4]	0.0±0.0[4]	0.0±0.0[4]	0.0±0.0[4]	--	--	0.0±0.0[4]
Control	2.0±1.3 b	74.9±5.9 a	32.5±2.4 a	31.7±3.4 a	64.2±5.3 a	0.49±0.02	85.5±2.0	97.9±2.1 a
F	9.76	96.93	123.67	63.34	93.15	--	--	93.89
P value	0.0058	<0.0001	<0.0001	<0.0001	<0.0001	--	--	<0.0001
Annona muricata								
Leaves	4.0±1.6 b	32.6±4.1 b	11.1±1.6 c	9.2±1.2 b	20.3±2.6 b	62.4±1.9	0.46±0.03	49.8±4.2 b
Branches	0.0±0.0[4]	68.2±5.2 a	22.1±2.1 b	22.0±2.4 a	44.1±4.0 a	64.4±2.1	0.50±0.02	97.6±1.0 a
Seeds	95.0±2.7 a	0.1±0.1 c	0.0±0.0[4]	0.0±0.0[4]	0.0±0.0[4]	--	--	0.0±0.0[4]
Control	1.0±1.0 b	80.5±5.1 a	29.9±2.1 a	26.7±2.4 a	56.6±3.4 a	70.9±2.2	0.46±0.02	96.7±1.8 a
F	138.33	125.81	23.47	21.75	30.36	2.26	1.12	67.61
P value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.1521 [ns]	0.3450 [ns]	<0.0001
Annona reticulata								
Leaves	6.0±2.2 b	31.2±4.0 b	15.3±2,1 b	12.7±1.7 b	26.4±3.3 b	84.9±2.5	0.48±0.04	52.1±5.2 b
Branches	6.0±2.7 b	61.9±5,9 a	27.5±3,1 a	27.1±2.6 a	54.6±5.3 a	87.4±2.1	0.50±0.02	67.6±5.4 b
Seeds	71.0±4.3 a	13.9±3,1 c	2.4±0,7 c	1.9±0.7 c	4.3±1.2 c	34.7±6.3[5]	0.37±0.1[5]	11.5±2.7 c
Control	5.0±3.1 b	78.3±6,8 a	34.3±3,9 a	32.3±2.8 a	66.6±6.3 a	84.7±1.2	0.49±0.02	90.9±2.2 a
F	45.24	34.17	38.86	52.97	55.11	0.63	0.79	54.36
P value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.5378 [ns]	0.1901 [ns]	<0.0001

[1]Means followed by different letters within columns indicate significant differences between treatments (GLM with a quasi-binomial distribution followed by Tukey's *post hoc* test, $p< 0.05$).
[2]Means followed by different letters within columns indicate significant differences between treatments (GLM with a quasi-Poisson distribution followed by Tukey's *post hoc* test, $p< 0.05$).
[3]Means followed by different letters within columns indicate significant differences between treatments (GLM with a binomial distribution followed by Tukey's *post hoc* test, $p< 0.05$).
[4] Not included in the analysis (null variance).
[5] Not analyzed due to small sample unit.
[ns] Not significant ($p>0.05$).

RESULTS

The process of extracting chemical compounds from *Annona* species with ethanol resulted on a wide range of yields. They oscilated from 0.99% (branches of *A. mucosa*) to 27.85% (seeds of *A. reticulata*). In a general way, the extracts from seeds presented a higher yield than the extracts from leaves and branches. Probably this is due to the high content of oils in *Annona* seeds (Amador *et al*., 1997).

The ethanolic extracts from seeds of *A. mucosa* and *A. montana* killed 100% of *Z. subfasciatus* adults at 1,500 mg kg^{-1} (mg extract per kg of bean grains) whereas the ethanolic extracts from seeds of *A. muricata* and *A. reticulata*, at this concentration, promoted 95% and 71% mortality, respectively (Table 2). On the other hand, the ethanolic extracts from *Annona* branches did not promote significant mortality; and only the ethanolic extract from leaves of *A. mucosa* slightly killed *Z. subfasciatus* adults (Table 2). Therefore, it is possible to infer that the four tested *Annona* species tend to accumulate a higher concentration of insecticidal compounds in their seeds. The ethanolic extracts from the seeds of *Annona* species also promoted sublethal effects on *Z. subfasciatus* better than the ethanolic extracts from branches and leaves (Table 2). The ethanolic extracts from the seeds of *A. mucosa* and *A. muricata* reduced to zero the number of eggs on beans resulting on zero damages on them (Table 2). Similarly, ethanolic extracts from seeds of *A. montana* and *A. reticulata* almost completely reduced the number of eggs, reducing the F_1 progeny and damages on bean grains (Table 2).

Based on these bioassays with *Annona* ethanolic extracts, the extracts from the seeds of *A. mucosa*, *A. montana*, *A. muricata* and *A. reticulata* were selected to estimate their LC_{50} and LC_{90}. The ethanolic extract from *A. mucosa* presented the lowest LC_{50} and LC_{90} among all *Annona* species [581.72 and 747.70 mg kg^{-1}, respectively (Table 3)]. It was followed by *A. reticulata* with an LC_{50} of 776.80 mg kg^{-1} and LC_{90} of 994.64 mg kg^{-1} while *A. montana* and *A. muricata* did not differ from each other based on the overlapping of their confidence intervals (Table 3). Moreover, the lethal concentrations varied according to *Z. subfasciatus* sex, and females supported higher doses than males (Table 3).

Table 3. Estimation of LC_{50} and LC_{90} (mg kg^{-1}) of ethanolic extracts from seeds of *Annona* species against *Zabrotes subfasciatus*, after five days of insect exposure.

Plant species	Sex	n [1]	Slope (± SE)	LC_{50} (95% CI) [2]	CL_{90} (CI) [2]	χ^2 [(3)]	d. f. [4]	h. [5]
Annona montana	Female	400	8.66±1.07	1,323 (1,214 - 1,409)	1,820 (1,718 – 1,960)	8.86	5	1.77
	Male	400	8.73±0.95	1,146 (1,062 - 1,218)	1,573 (1,481 – 1,694)	7.41	5	1.48
	Male + Female	800	10.94±1.26	1,206 (1,154 - 1,255)	1,553 (1,473 – 1,675)	1.88	4	0.47
Annona mucosa	Female	400	12.68±1.65	623.66 (589.92 - 651.37)	775.56 (739.84 – 827.97)	8.87	5	1.77
	Male	400	7.11±1.28	512.12 (416,85 - 570,52)	755.30 (704.50 – 820.44)	10.63	6	1.77
	Male + Female	800	11.01±0.91	581.72 (560.85 - 600.45)	747.70 (720.85 – 782.42)	2.21	4	0.55
Annona muricata	Female	400	11.77±1.71	1,263.03 (1,181.4 -1,329.5)	1.597.25 (1,509.5 - 1,734.9)	4.70	4	1.18
	Male	400	9.84±1.23	1,098 (1,028 - 1,158)	1,454 (1,369 - 1,580)	0.61	4	0.15
	Male + Female	800	10.22±0.98	1,177 (1,123 - 1,224)	1,542 (1,476 - 1,632)	3.66	4	0.91
Annona reticulata	Female	400	18.29±3.03	848.32 (809.49 - 875.32)	986.69 (953.93 - 1.041.41)	1.59	4	0.40
	Male	400	9.27±1.52	712.51 (630.15 - 765.37)	960.07 (910.58 - 1,029.80)	1.79	5	0.36
	Male + Female	800	11.18±1.72	776.80 (723.44 - 810.89)	994.64 (957.96 - 1,055.13)	5.44	4	1.36

[1] n: number of insects tested.
[2] CI: confidence interval ($p < 0.05$).
[(3)] χ^2: calculated chi-square value.
[4] d. f.: degrees of freedom.
[5] h.: factor of heterogeneity.

Table 4. Lethal and sublethal effects (means ±SE) of fractions prepared from ethanolic extract of *Annona mucosa* seeds at previous estimated LC_{50} (581.58 mg kg^{-1}) against *Zabrotes subfasciatus*.

Fraction	Mortality	nº eggs/ sample[1]	F_1 Progeny[1]			Viability (%) (egg-adult) [2]	Sex ratio	Damages on grains (%)[2]
			Males	Females	Total			
Hexane	100.0±0.0[3]	0.0±0.0 [3]	0.0±0.0 [3]	0.0±0.0 [3]	0.0±0.0 [3]	-	-	0.0±0.0 [3]
Hydro-methanol	20.0±5.6	10.1±2.5b	1.7±0.8b	1.8±0.6b	3.5±1.3b	27.3±8.6b	0.34±0.11[4]	5.9±1.8
Control	0.0±0.0 [3]	99.7±7.2a	34.3±3.2a	37.2±3.5a	71.5±4.7a	72.3±2.2a	0.52±0.03	85.8±2.4
F	-	146.93	108.73	145.77	184.81	25.37	-	286.18
Value of *p*	-	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	-	<0.0001

[1]Means followed by different letters within columns indicate significant differences between treatments (GLM with a quasi-binomial distribution followed by Tukey's *post hoc* test, $p< 0.05$).
[2]Means followed by different letters within columns indicate significant differences between treatments (GLM with a quasi-Poisson distribution followed by Tukey's *post hoc* test, $p< 0.05$).
[3] Not included in the analysis (null variance).
[4] Not analyzed due to small sample unit.
[ns] Not significant (p>0,05).

Because of its higher toxicity to *Z. subfasciatus*, the ethanolic extract from seeds of *A. mucosa* was fractionated. It was obtained one hexane fraction and other methanol:water fraction 8:2 (v v^{-1}). Both the hexane and hydro-methanol fractions from the ethanolic extract from seeds of *A. mucosa* promoted mortality of *Z. subfasciatus* adults (Table 4). However, the higher insecticidal effect was caused by the hexane fraction that killed 100% of *Z. subfasciatus* adults (Table 4). The same trend was observed when the sublethal effects were evaluated; the hexane phase promoted the most pronounced effects. It completely reduced the number of eggs, F_1 progeny and damages on bean grains (Table 4). Although less accentuated, the hydro-methanol phase also caused significant sublethal effects, which demonstrates the presence of bioactive compounds with different polarities in *A. mucosa* seed extract (Table 4).

DISCUSSION

The ethanolic seed extracts from *A. montana, A. mucosa, A. muricata and A. reticulata*, applied at 1,500 ppm, promoted lethal and sublethal effects on *Z. subfasciatus*. They killed adults of *Z. subfasciatus*, reduced the eggs on beans grains, reduced the F_1 progeny and the damage on grains. Based on such results they can be considered as promising grain protectors. The best one was the extract from *A. mucosa*, which promoted 100% of mortality of *Z. subfasciatus* and completely inhibited the oviposition, the F_1 progeny and damages on grains (Table 2). Crude extracts from *Annona* seeds may be efficient to control Bruchinae beetles and a source of different insecticidal compounds. Extracts from seeds of *Annona squamosa* L. promote insecticidal effects against *Callosobruchus chinensis* L. (Coleoptera: Chrysomelidae) and *Callosobruchus maculatus* Fabricius (Coleoptera: Chrysomelidae) (Ohsawa *et al.*, 1990; Al Lawati *et al.*, 2002). The bioactivity of *A. squamosa* against *C. chinensis* was attributed to the acetogenin trihydroxy-bistetrahydrofuran fatty acid α,β-unsaturated γ-lactone (Ohsawa *et al*., 1990). The seed extract from *A. sylvatica* seeds is toxic to *Z. subfasciatus*, at 1,500 mg kg^{-1} it inhibited oviposition and F_1 progeny of *Z. subfasciatus* (Gonçalves *et al.*, 2015). Similarly, in the present study the seed extracts were the most active ones, they promoted both lethal and sublethal effects (Table 2). The seed extract from *A. mucosa* completely inhibited oviposition and F_1 progeny (Table 2). LC_{50} of *A. mucosa* (LC 50: 581.72 mg kg^{-1}) was lower than the one estimated for *A. sylvatica* (LC_{50}: 729.55 mg kg^{-1}) by Gonçalves *et al.* (2015). Differences in the bioactivity among ethanolic extracts obtained from the different species and structures of *Annona* are possibly associated with qualitative and quantitative variations in their chemical profiles at harvesting time. This occurs because the plant secondary metabolites have an idiosyncratic distribution both taxonomically and ontogenically (Berenbaum, 1995). Such variations in the concentration of active ingredients in the ethanolic extracts might reduce or increase their efficacy to control *Z. subfasciatus*. Nonetheless, all seed extracts tested against *Z. subfasciatus* promoted mortality above 70% and are suitable to produce a botanical insecticide (Table 2).

The high efficiency of *A. mucosa* ethanolic extract and fractions to control *Z. subfasciatus* place such species as an alternative to formulate a botanical insecticide (Tables 2, 3 and 4). However, some of the challenges to produce a botanical insecticide are the availability of botanical raw material to attend the market demand and the qualitative and quantitative variation of insecticidal compounds in plants that can reduce its controlling efficiency (Isman, 1997). Because *Annona* seeds are a residue produced during the process of extracting pulp from fruits, they can be used as raw material to produce botanical insecticides and provide a new source of income to the industry. Moreover, the presence of bioactive compounds in *Annona* seeds associated with their high yield of extraction (up to 27.85%) make them a promissory source to formulate botanical insecticides suitable to control populations of *Z. subfasciatus* in warehouses. The concentration of the hexane and hydro-methanol fractions of *A. mucosa*

(581.78 g/ton of grain) or ethanolic extracts (1,500 g extract ton^{-1} of grain), is similar to the concentration recommended for K-Obiol 2P (2 g a.i.kg^{-1}), 250-1,000 g of product ton^{-1} of grain. This indicates the economically feasibility of adopting *Annona* species as sources of botanical insecticides; and a process of chemical standardization of active ingredients in the *Annona*-based insecticide could fix the problem with efficiency variation. An ethanolic extraction is a simple and efficient method of extracting insecticidal compounds from seeds of *A. mucosa* to control *Z. subfasciatus*, and a simple fractionation procedure can improve its efficacy (Tables 2 and 4). This method may be suitable for small organic farmers produce their own botanical insecticide.

Previous studies revealed acetogenins and triglycerides as major compounds in the ethanolic extract from seeds of *A. mucosa*, respectively (Ribeiro *et al*., 2013). The spectra of the chemical bioactive fractions obtained by proton nuclear magnetic resonance (^{1}H NMR) revealed triglycerides as the major chemical constituent in the hexane partition and acetogenins in the hydro-methanol one (Ribeiro *et al*., 2013). In addition, rolliniastatin-1 is the major acetogenin in *A. mucosa* seeds (Ansante *et al*., 2015; Souza *et al*., 2017). Many acetogenins have been isolated and identified in species of *Annona* (Bermejo, 2005). Acetogenins tend to affect insect respiration (Bermejo, 2005). For example, the acetogenin annonin present in *Annona squamosa* L. (Annonaceae) inhibits mitochondrial complex III (Pavela, 2016). This is a very interesting mode of action because the only non-fumigant insecticide registered in Brazil for controlling *Z. subfasciatus* is K-Obiol 2P, a deltamethrin-based insecticide that presents a neurotoxic mode of action (Agrofit, 2018). The compound deltamethrin act as a modulator of voltage gated sodium channels in the neurons of insects (Nauen *et al*., 2011). On the other hand, thyglicerides (present in the hexane fraction) also promote neurotoxicity on insects, they can inhibit the activity of acetylcholinesterase (Perumalsamy *et al*., 2015). Therefore, a botanical insecticide based on *A. mucosa* seeds might be a promissory tool to control populations of *Z. subfasciatus* infesting warehouses.

CONCLUSION

Ethanolic extracts and semi-purified fractions from seeds of *Annona* species can promote lethal and sublethal effects on *Z. subfasciatus*; especially from seeds of *A. mucosa*. Therefore, seed residue, from the food industry, might be applied for the formulation of a botanical insecticide to control Mexican bean weevil's populations at warehouses.

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Compliance with ethical standards. It does not apply.

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