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ORIGINAL PAPER

Lethal and growth inhibitory activities of Neotropical Annonaceae-derived extracts, commercial formulation, and an isolated acetogenin against *Helicoverpa armigera*

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Abstract Among tropical plant families, members of Annonaceae have great potential as a source of biopesticides. To develop an alternative tool for control of *Helicoverpa armigera*, efficacy of ethanolic extracts from seeds of five species belonging to the genus *Annona* (*A. montana* Macfad., *A. mucosa* Jacq., *A. muricata* L., *A. reticulata* L. and *A. sylvatica* A. St.-Hil.) and an acetogenin-based commercial bioinsecticide (Anosom® 1 EC, 10,000 ppm of annonin as the main active ingredient) were evaluated in a dietary exposure bioassays. In an initial screening, an ethanolic extract from *A. mucosa* seeds (LC_{50} = 1479 ppm) and Anosom® 1 EC (LC_{50} = 1151 ppm) were the most promising treatments. In addition to acute toxicity, pronounced inhibition of *H. armigera* larval growth was observed in both treatments. Using chromatographic techniques, bioguided fractionations were conducted and the acetogenin bis-tetrahydrofuran rolliniastatin-1 was isolated as the primary compound from the most active fractions of *A. mucosa*. At a concentration of 41.55 ppm, rolliniastatin-1 caused total mortality of *H. armigera* larvae after the fourth day of exposure. In greenhouse trials, extract of *A. mucosa* (as an emulsifiable concentrate formulation) and the botanical insecticide based on extract of *Annona squamosa* L. (Anosom® 1 EC), both at LC_{90} values previously estimated, were compared with a diamide-based commercial insecticide (flubendiamide 480 SC) for mortality after 168 h of exposure to larvae on tomato plants; all treatments caused high larval mortality (>90%). Thus, the results of this study indicate that the derivatives of Annonaceae are a useful alternative for the integrated management of *H. armigera*.

Keywords Annonaceae · Acetogenin · Botanical insecticides · Old world bollworm · Rolliniastatin-1 · Chromatographic techniques

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Key message

- Annonaceae species were screened to detect sources of insecticidal compounds.
- An ethanolic extract from *A. mucosa* seeds caused strong mortality to *Helicoverpa armigera*.
- The major compound present is the acetogenin bis-tetrahydrofuran rolliniastatin-1.
- Ethanolic extract from *A. mucosa* seeds also caused high larval mortality in greenhouse trial.

Introduction

Helicoverpa armigera (Hübner, 1808) (Lepidoptera: Noctuidae) is a polyphagous pest species whose larvae damage vegetative and reproductive structures of several crops

(Lammers and Macleod 2007) in Africa, Asia, Oceania and Europe (Venette et al. 2003), and more recently in South America (Czepak et al. 2013; Murúa et al. 2014). The global agricultural production losses caused by *H. armigera* may approach USD 5 billion annually, and in China and India, an estimated 50% of pesticide use is for control of this pest (Lammers and Macleod 2007). The first records of *H. armigera* in Brazil were on soybean in Bahia and cotton in Mato Grosso during the 2012/2013 harvests (Czepak et al. 2013). Currently, this pest is widespread throughout Brazil, attacking cotton, soybean, corn, millet, tomato, wheat (Czepak et al. 2013), citrus (Bueno et al. 2014) and forage crops (Bueno and Sosa-Gómez 2014).

In Brazil, chemical control with synthetic insecticides is the primary method for management of *H. armigera*; however, most of the insecticides used for its control are registered for emergency use (Czepak et al. 2013). Furthermore, *H. armigera* has many documented cases of resistance to insecticides, including pyrethroids, organophosphates, carbamates, organochlorines (Jouben et al. 2012), spinosins (Aheer et al. 2009) and toxins derived from *Bacillus thuringiensis* (Zhang et al. 2011; Yang et al. 2013); thus, the motivation for studies to identify alternative pest control methods is provided.

The use of plants with insecticidal activity can be an effective strategy as part of an integrated pest management program, also in most of cases these compounds have low toxicity to mammals and non-target organisms and low persistence in the environment; their use is environmentally sound (Cloyd 2004; Isman 2006). Moreover, plants with insecticidal activity constitute an important option for pest management in organic food production systems in which synthetic compounds are not permitted (Zanardi et al. 2015).

Among tropical plant families, the Annonaceae has great potential as a source of biopesticides and has been highlighted from plant systematics in the last decades (Isman 2006; Isman and Seffrin 2014). The family includes 135 genera and approximately 2500 described species to date, with most occurring in the pantropical region (Chatrou et al. 2004). Among the classes of bioactive compounds found in Annonaceae, the acetogenins have attracted attention since the 1980s because the structural characteristics that promote a wide range of biological activities, including potent insecticidal action (Ocampo and Ocampo 2006). Acetogenins are a series of natural products (C-35/C-37) derived from long-chain fatty acids combined with a unit of 2-propanol (Alali et al. 1999). These molecules are potent inhibitors of complex I (NADH: ubiquinone oxidoreductase) of the mitochondrial electron-transport system and of the coenzyme NADH in the cell membranes of target arthropods (Lewis et al. 1993).

In recent research, the derivatives of Annonaceae were effective against different pest species of agricultural importance, including *Plutella xylostella* L. (Leatemia and Isman 2004; Trindade et al. 2006), *Trichoplusia ni* Hübner and *Myzus persicae* (Sulzer) (Ribeiro et al. 2014a), *Panonychus citri* (McGregor) (Ribeiro et al. 2014b), *Spodoptera frugiperda* (JE Smith) (Blessing et al. 2010; Tolosa et al. 2014; Ansante et al. 2015a), *Diaphorina citri* Kuwayama (Ribeiro et al. 2015) and coleopteran stored grains pests (Ribeiro et al. 2013, 2014c; Gonçalves et al. 2015). However, until this study, the effectiveness of extracts of Neotropical Annonaceae on *H. armigera* has not been examined. Thus, in this study, first, the bioactivity of seed ethanol-extracts from five species of *Annona* (*A. mucosa, A. muricata, A. montana, A. reticulata* and *A. sylvatica*) and the acetogenin-based commercial bioinsecticide [Anosom® 1 EC (Agrilife SOM Phytopharma Ltd., Hyderabad, Andhra Pradesh State, India)] was evaluated on *H. armigera* in laboratory bioassays. Second, bioguided fractionation was conducted to isolate the primary acetogenin of the most promising extract, which was evaluated for the toxicity on *H. armigera*. Finally, the efficacy of the most promising extract (as an emulsifiable concentrate formulation) was compared with that of acetogenin-based and synthetic-based commercial insecticides for the control of *H. armigera* on tomato plants in a greenhouse trial.

Materials and methods

Plant material and preparation of plant extracts

Voucher specimens of the species used in this study (Table 1), previously identified by Prof. Dr. Renato Mello-Silva [Department of Botany, Biosciences Institute/University of São Paulo (IB/USP)], were deposited in the herbarium of the Department of Biological Sciences at "Luiz de Queiroz" College of Agriculture/University of São Paulo in Piracicaba municipality, São Paulo State, Brazil.

To prepare the extracts, seeds collected from ripe fruit were dried in a forced-air oven at 40 °C for 48–72 h. The dried seeds were ground in a Wiley mill to a fine powder, which were stored in sealed glass in a domestic freezer (−10 °C) until use.

The organic extracts were obtained by maceration in solvent ethanol (at the ratio of 1:5 w/v). The powder and solvent stood in hermetically sealed flasks for three days, followed by filtration with filter paper. The process of soaking in ethanol was repeated three times for each sample. The remaining solvent in the filtered solution was eliminated in a rotaevaporator at 50 °C and a pressure of −600 mmHg. After complete solvent evaporation in an

Table 1 Collection data for the species of Annonaceae used in the study

Species	Collection site	Collection date	Voucher
Annona montana Macfadyen	ESALQ/USP Campus, Piracicaba, SP, Brazil (22°42′28,2″S; 47°37′59,4″W; elevation: 537 m)	21/03/11	121,203
Annona mucosa Jacquin	ESALQ/USP Campus, Piracicaba, SP, Brazil (22°42′28,5″S; 47°37′59,6″W; elevation: 534 m)	17/03/11	120,985
Annona muricata Linnaeus	ESALQ/USP Campus, Piracicaba, SP, Brazil (22°42′25,4″S; 47°37′43,9″W; elevation: 576 m)	12/04/11	121,892
Annona reticulata Linnaeus	São Luís Farmer, Descalvado, SP, Brazil (21°52′58,0″S; 47°40′38,0″W; elevation: 679 m)	02/04/11	123,318
Annona sylvatica A. St.-Hil.	Emilio Falcão Avenue, Erval Seco, RS, Brazil (27°25′41,8″S; 53°34′11,2″W; elevation: 466 m)	25/04/11	121,205

airflow chamber, extraction yield was determined for the seeds of each species.

Test insects

The colony of *H. armigera* used in the bioassays was established from adults collected in soybean and corn crops in the state of São Paulo, Brazil. Dr. Alexander Specht (Embrapa Cerrado, Brasilia, DF, Brazil) previously identified the species using morphological and molecular characters described in the protocol proposed by Specht et al. (2013).

Stock rearing of *H. armigera* was kept under controlled laboratory conditions (temperature: 25 ± 2 °C; RH: 60 ± 10%; photoperiod: 14 L:10 D h), with the caterpillars fed on an artificial diet (Greene et al. 1976). Adults were fed honey water solution 10% (w/v). Reproduction of *H. armigera* was conducted following the protocols of Kao (1995) and Jah et al. (2012).

Bioassays

The laboratory tests were conducted under controlled conditions (temperature: 25 ± 2 °C; RH: 60 ± 10%; photoperiod: 14 L:10 D h), whereas the greenhouse trial (average temperature: 24.3 °C, with a maximum of 25.6 °C and a minimum of 16.7 °C; average RH: 58%, with a maximum of 95% and a minimum of 38%; natural light) was conducted during June 2015 in Botucatu, SP, Brazil.

Screening of *Annona* seed extracts in the laboratory

To identify the most promising species for control of *H. armigera*, preliminary tests with ethanolic seed extracts prepared from five species *Annona* (*A. muricata*, *A. mucosa*, *A. reticulata*, *A. sylvatica* and *A. montana*) and an acetogenin-based commercial bioinsecticide (Anosom® 1 EC) were conducted. The extracts were solubilized in a mixture of acetone/methanol (1:1 v/v) and added 5 mL per 300 g of diet as described by Ansante et al. (2015). Anosom® was dissolved in distilled water and added to the diet at identical proportion as that for the extracts.

The treatments were incorporated into artificial diet (Greene et al. 1976) at concentration of 2000 ppm (diagnostic concentration). As negative controls, we used deionized water and the solvent solution [acetone/methanol (1:1, v/v)], which were used to solubilize the biopesticide and extracts, respectively, at identical proportions.

Treatments (1.5 mL of each treated diet) were placed in Elisa plates (TPP® Techno Plastic Products AG, Trasadingen, Canton Schaffhausen, Switzerland) using a micropipettor. Twenty-four hours after the addition, each cell was infested with neonate *H. armigera* larvae (Talekar et. 2006; Tolosa et al. 2014). Six replicates were used for each treatment, and each replicate was one plate ($n = 144$). Larval mortality rates were quantified daily for 7 days.

Concentration–response curves of active extracts

Based on the results from the screening, the most promising treatment (ethanolic seed extract from *A. mucosa*) and the acetogenin-based commercial bioinsecticide (Anosom® 1 EC) were evaluated again to estimate the LC_{50} and LC_{90} (the concentrations required to kill 50 and 90 percent of exposed caterpillars, respectively) and the EC_{50} (the concentration required to reduce the larval weight by 50%). Therefore, five concentrations were defined for each concentration (range 0–2000 ppm), based on Finney (1971) procedure.

The experimental procedures were identical to those used in the screening tests. Four replicates were used for each treatment, and each replicate consisted of a single plate ($n = 96$).

Average lethal time (LT_{50})

The LT_{50} values (the time required to kill 50% of test population) for the Anosom® 1 EC and ethanolic extract from *A. mucosa* seeds (most promising extract) were

estimated at concentrations of 1000 and 2000 ppm. The experimental procedures were identical to those used in the screening tests.

Isolation of and bioassay with the primary acetogenin in the selected seed extract

Chromatographic procedures were used to purify and isolate the primary acetogenin in the ethanolic extract from *A. mucosa* seeds. For this purpose, we used various chromatography techniques, which included adsorption chromatography on an analytical thin layer (ADCC) using aluminum sheet silica gel 60 F_{254} 0.2 mm thick, adsorption column chromatography (CC) using as stationary phase silica gel 70–230 mesh and 230–400 mesh, and high-performance liquid chromatography (HPLC). For HPLC, a gas chromatograph model 1200 (Agilent Technologies) equipped with quaternary pump G1311A, degasser G1322A, automatic sampler G1329A and ultraviolet detector G1314B was used. The equipment was coupled to an interface G1369A, and the chromatograms were recorded by EZCrom Ellite software. The stationary phase was a reversed phase Phenomenex Luna C-18 for analytic (10 micrometers, 25.0 × 0.46 cm) and preparative (10 micrometers, 25.0 × 1.0 cm) purposes with a recycle valve and 200 µL loop.

The characterization of the isolated compound was conducted by spectroscopic techniques [dimensional (^{1}H NMR and ^{13}C) and two-dimensional (COSY, HSQC, HMBC) nuclear magnetic resonance] and by mass spectrometry. For these characterizations, spectrometers Bruker Avance III NanoBay 9,4T (400 MHz for ^{1}H NMR and 100 MHz for ^{13}C NMR) and Micromass Quattro LC, with methods for direct insertion and ionization mode to capture electrons (negative mode), were used.

To evaluate the insecticidal effect of the isolated acetogenin against *H. armigera*, we used a concentration equivalent to 10% of the estimated LC_{50} value for the respective seed ethanol-extract. The experimental procedures were identical to those used in the screening tests. Four replicates were used for each treatment, and each replicate was one plate ($n = 80$).

Bioactivity of selected extracts compared with a commercial insecticide and acetogenin-based commercial bioinsecticide in greenhouse trials

To evaluate the efficacy of the most promising extract and Anosom® 1 EC, under greenhouse conditions, a test was performed using a commercial insecticide based on flubendiamide (Belt® 480 SC, Bayer Corporation, Dormagen, Germany) as a positive control. The insecticide Belt® 480 SC was used at the dose recommended by the manufacturer (416.7 ppm), whereas the ethanolic extract from *A. mucosa* seeds and the Anosom® 1 EC were used at the LC_{90} values (1479 and 1151 ppm, respectively) estimated previously. For preparation of aqueous emulsified formulation, the *A. mucosa* ethanolic seed extract was solubilized in the organic solvents acetone/methanol (1:1, v/v) (100 g L^{-1}), with the subsequent addition of Tween® 80 emulsifier at a concentration of 10 g L^{-1}.

The test was conducted on tomato plants 30–45 days old. Each plant was planted in a 2.5-L pot containing soil and commercial substrate (Plantmax®) mixed at a ratio of 3:1 (v/v). The treatments were applied to plants with a gravity-type spray gun (Model Arprex 5A), coupled to an air compressor set to provide a pressure of 0.5 kgf cm^{-2}. The plants were sprayed until the point of runoff, and after the residues dried, a leaf from each plant was artificially infested with 10 neonate *H. armigera* larvae. Subsequently, a "voile" cage was placed on the leaves to prevent insect escape. Ten replicates were used for each treatment, and each replicate was one plant ($n = 100$).

The infested leaf remained on countertops in a greenhouse. After 6 days of infestation, mortality and weight of the surviving larvae were determined. Larvae were considered dead when there was no reaction (movement) after a light touch with a fine brush.

Data analyses

Generalized linear models of the exponential family of distributions (Nelder and Wedderbum 1972) were used for the analyses of studied variables. The verification of quality adjustment was performed through the half-regular graph odds with simulation envelope (Demétrio and Hinde 1997; Hinde and Demétrio 1998). When there were significant differences among treatments, multiple comparisons (Tukey's test, $p < 0.05$) were performed using the glht function by means of Multicomp package, with adjustment of p values. All analyses were performed using the "R" statistical software version 2.15.1 (R Development Core Team 2012).

A binomial model with a complementary log–log link function (gompit model) was used to estimate the lethal concentrations (LC_{50} and LC_{90}), using the *Probit Procedure* in the software SAS version 9.2 (SAS Institute 2011). Finally, the mean lethal time (LT_{50}) was estimated using the method proposed by Throne et al. (1995) for Probit analysis of correlated data.

Results

Extraction yield

The extraction yield varied significantly (11–27.85%), depending on the species (Table 2). The highest yield was

Table 2 Yields of extracts obtained by maceration of seeds in ethanol (1:5, w/v) from the five species of Neotropical Annonaceae

Species	Material weight (g)	Yield	
		(g)	(%)
Annona montana	95.60	19.34	20.23
Annona mucosa	106.21	19.95	18.79
Annona muricata	104.50	22.06	21.10
Annona reticulata	100.00	27.85	27.85
Annona sylvatica	100.00	11.00	11.00

obtained from seeds of *A. reticulata* and the lowest from seeds of *A. sylvatica*.

Laboratory bioassays

Significant differences were detected among the treatments tested at 2000 ppm in the initial screening (Table 3). After 168 h of exposure (bioassay endpoint), Anosom® 1EC [LC_{50} = 312.08 ppm; Table 4] and the *A. mucosa* seed extract [LC_{50} = 411.55 ppm; Table 4] were the most promising treatments, resulting in total mortality of exposed larvae (Table 3). The effect of seed extracts from *A. reticulata*, *A. montana* and *A. muricata* on larvae was different from that of the control (Table 3), but the effects were much less pronounced than those of the more active treatments.

The average lethal time (LT_{50}) estimated for the most active treatment varied according to the concentration tested and was 45.15 h (95% CI = 7.66–88.40 h; χ = 11.23; g.l. = 4; n = 672) and 34.95 h (95% CI = 27.54–41.63 h; χ = 6.87; g.l. = 4; n = 672) for *A. mucosa* ethanolic seed extract at concentrations of 1000 and 2000 ppm, respectively. For Anosom® the estimated average lethal times were 47.69 h (95% CI = 18.51–76.22; χ = 10.19; g.l. = 4; n = 672) and 39.78 h (95% CI = 18.42–56.20; χ = 17.52; g.l. = 4; n = 672) for concentrations of 1000 and 2000 ppm, respectively.

In addition to acute effects, Anosom® [EC_{50} = 173.60 - ppm (95% CI = 56.82–290.40 ppm; n = 324)] and *A. mucosa* ethanolic seed extract [EC_{50} = 239.00 ppm (95% CI = 129.10–348.00 ppm; n = 367)] significantly inhibited *H. armigera* larval development. However, the difference between these treatments was not significant based on the comparison of the confidence intervals of the adjusted values.

Using different chromatography techniques, the primary compound of the *A. mucosa* ethanolic seed extract was isolated and then was identified as acetogenin bis-tetrahydrofuran rolliniastatin-1 (Fig. 1) using spectroscopic and spectrometric techniques. The isolated acetogenin increased the larval mortality after 48 h of exposure, and total mortality was observed after 120 h of exposure (Table 5).

Greenhouse trial

Larval mortality exceeded 90% with exposure to *A. mucosa* ethanolic seed extract and the Anosom® 1 EC (Table 6).Total mortality of larvae was observed with the

Table 3 Accumulated mortality (±SE) of *Helicoverpa armigera* caterpillars exposed to artificial diet treated (2000 ppm) with various derivatives of Annonaceae

Treatments	Exposure time (h)[1]			
	24	72	120	168
E.E.S. *Annona reticulata*	4.35 ± 2.20	27.66 ± 3.16	29.11 ± 2.96	29.36 ± 3.02
E.E.S. *Annona mucosa*	31.55 ± 5.08	100.00 ± 0.00*	100.00 ± 0.00*	100.00 ± 0.00*
E.E.S. *Annona montana*	0.00 ± 0.00*	21.55 ± 3.04	24.62 ± 3.30	24.62 ± 3.30
E.E.S. *Annona muricata*	6.05 ± 2.32	35.77 ± 2.88	35.77 ± 2.88	35.77 ± 2.88
E.E.S. *Annona sylvatica*	0.69 ± 0.69	7.01 ± 2.03	7.01 ± 2.03	7.01 ± 2.03
Anosom® 1 EC	97.92 ± 1.42	100.00 ± 0.00*	100.00 ± 0.00*	100.00 ± 0.00*
Control (water)	0.00 ± 0.00*	0.72 ± 0.72	1.42 ± 0.90	2.14 ± 1.47
Control (Acet/meth, 1:1)	2.17 ± 1.48	2.84 ± 1.79	2.84 ± 1.79	2.84 ± 1.79
	$F_{5.30}$ = 81.827; $p < 0.0001$	$F_{5.30}$ = 27.507; $p < 0.0001$	$F_{5.30}$ = 29.588; $p < 0.0001$	$F_{5.30}$ = 25.398; $p < 0.0001$

E.E.S. Seed ethanol-extract

* Not included in the statistical analysis (null variance)

[1] Means followed by different letters within a column indicate significant differences among treatments (GLM with quasi-binomial distribution, followed by post hoc Tukey's test, $p < 0.05$)

Table 4 Estimates of LC_{50} and LC_{90} (ppm) with confidence intervals for the acetogenin-based commercial bioinsecticide (Anosom® 1 EC) and *Annona mucosa* ethanolic seed extract—ESAM (Annonaceae) on *Helicoverpa armigera* caterpillars after 168 h of exposure

Treatments	n	Slope ± EP (p value)	LC_{50} (CI)	LC_{90} (CI)	χ^2	d.f.	h.
Anosom®	672	2.11 ± 0.21 ($p < 0.0001$)	312.08 (244.00–377.45)	1151.00 (956.83–1457.00)	4.69	3	1.56
ESAM	672	2.16 ± 0.37 ($p < 0.0001$)	411.55 (185.07–609.85)	1479.00 (1011.00–3069.00)	9.18	4	2.29

n number of insects tested

CI confidence interval at 95% error probability

χ^2 Pearson's Chi-square value

df degrees of freedom

h heterogeneity factor

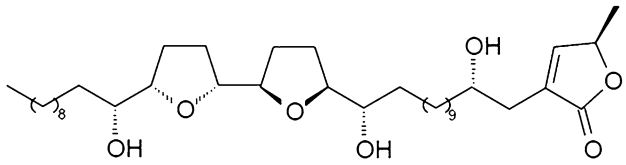

Fig. 1 Chemical structure of acetogenin rolliniastatin-1, the primary compound of the ethanol extract of *Annona mucosa* seeds (ESAM)

flubendiamide-based commercial insecticide (Belt® 480 SC) (Table 6) during the exposure period.

In addition to high mortality rates, the average weight of surviving larvae was significantly reduced (Table 6), which confirmed that these derivatives of Annonaceae inhibit larval development, an effect previously detected in laboratory tests.

Discussion

The public awareness of the negative impacts on the environment and human health of the use of synthetic insecticides is founded (Isman and Grieneisen 2014). The widespread use of conventional insecticides and their negative effects on human health have stimulated the increase in botanical insecticide research as well as the identification of plant-derived compounds with insecticidal activity (Castillo-Sánchez et al. 2010; Baldin et al. 2015; Tak et al. 2016). Our bioassays presented the ethanolic extract from seeds of *A. mucosa* (ESAM) as a promising tool in pest management. ESAM produced 100% larval mortality, and its activity was comparable to flubendiamide and acetogenin-based insecticides under laboratory and greenhouse conditions. The high insecticidal activity of ESAM was also observed on *S. frugiperda* larvae where its efficiency was similar to a chlorantraniliprole-based insecticide and authors suggest their potential use in small farms due to simplicity of preparation (Ansante et al. 2015). The bioactivity of crude extracts is related to the interaction of different compounds which form complex mixtures (Isman 2000). ESAM bioactivity is associated with presence of alkaloids, triglycerides and especially acetogenins as well as synergistic interaction between these chemical compounds (Ribeiro et al. 2014c; Ansante et al. 2015).

Table 5 Cumulative mortality (% ± SE) of *Helicoverpa armigera* caterpillars exposed to an artificial diet containing the acetogenin bis-tetrahydrofuran rolliniastatin-1 (41.55 ppm) at different times of exposure

Treatment	Exposure time (h)[1]				
	24	48	72	96	120
Rolliniastatin-1	0.00 ± 0.00*	28.75 ± 3.75	61.25 ± 4.27	92.50 ± 3.23	100.00 ± 0.00*
Control (acetone)	0.00 ± 0.00*	1.25 ± 1.25	2.50 ± 1.44	2.50 ± 1.44	2.50 ± 1.44
Control (deionized water)	0.00 ± 0.00*	1.25 ± 1.25	1.25 ± 1.25	1.25 ± 1.25	1.25 ± 1.25
	–	$F_{2.9} = 24.99$; $p < 0.0001$	$F_{2.9} = 74.387$; $p < 0.0001$	$F_{2.9} = 118.61$; $p < 0.0001$	$F_{1.6} = 0{,}4081$ ^{ns}; $p < 0{,}5465$

ns not significant

* Not included in the analysis (null variance)

[1] Means followed by different letters within a column indicate significant differences among treatments (GLM with quasi-binomial distribution, followed by post hoc Tukey's test, $p < 0.05$)

Table 6 Efficacy of formulated ethanolic extract from *Annona mucosa* seeds and an acetogenin-based (Anosom® 1 EC) and flubendiamide-based (Belt® 480 SC) commercial insecticides against *Helicoverpa armigera* in a greenhouse trial

Treatments	Concentration (ppm)	Mortality[1] Mean ± SE	Corrected mortality[2]	Larval weight (mg)*
ESAM[3]	1479.0	95.00 ± 2.23	94.68	0.4875 (5)
Anosom® 1 EC	1151.0	93.00 ± 2.60	94.16	0.82 (7)
(Belt® 480 SC)	416.7	100.00 ± 0.00*	100.00	–
Control (deionized water)		4.44 ± 1.75	–	5.14 ± 0.05
Control (methanol/water, 1:10 (v/v) + Tween 80®, 0.5%		6.00 ± 2.21	–	5.01 ± 0.15
		$F_{3.36} = 113.25$; $p < 0.0001$		

* Data not analyzed because of small sample size. The value in parentheses represents the number of surviving caterpillars in those treatments

[1] Means followed by different letters within a column indicate significant differences among treatments (GLM with quasi-binomial distribution, followed by post hoc Tukey's test, $p < 0.05$)

[2] Corrected mortality calculated using the formula of Abbott (1925)

[3] *ESAM Annona mucosa* seed ethanol-extract

The potential insecticidal properties of Annonaceae crude extract species for the control of other lepidopteran pests of horticultural crops have been highlighted in recent studies. For example, the activity of *A. muricata* leaf-extract on cabbage leaves, at concentration of 5000 ppm causes 100% mortality of *P. xylostella* larvae (Trindade et al. 2006). Similarly, an ethanol extract of *A. squamosa* seeds applied to cabbage showed greater efficacy against *P. xylostella* than a rotenone-based or pyrethrin-based commercial insecticides (Leatemia and Isman 2004). Studies with four species of *Annona* (*A. montana*, *A. mucosa*, *A. muricata* and *A. sylvatica*), which were also investigated in our study, and the acetogenin-based commercial bioinsecticide found significant insecticidal activity on third instar *T. ni* larvae. The *A. mucosa* extract (ESAM) and the acetogenin-based commercial bioinsecticide cause high *T. ni* larval mortality (98%) after 120 h on cabbage leaves in a greenhouse trial, showing higher efficiency than that of a pyrethrin-based commercial insecticide (Insect Spray®). In our study, the mortality caused by ESAM extract and acetogenin-based commercial bioinsecticide was higher (100%) than that observed by those authors; however, we used neonate *H. armigera* larvae, which were likely more sensitive to the compounds than third instar *T. ni* larvae (Ribeiro et al. 2014a).

The chromatographic separation techniques mediated bioguided trials (Ansante et al. 2015) identified the acetogenin bis-tetrahydrofuran rolliniastatin-1 as the primary active component from ESAM. The toxic effect of nine acetogenins mono- and bis-tetrahydrofuran derived from *A. cherimolia* was evaluated on *S. frugiperda* and was observed that major acetogenin bis-tetrahydrofuran squamocin (annonin) was the most effective (Colom 2007). The promising ESAM activity reported in our study is probably related to its major compound rolliniastatin-1 that is composed of two tetrahydrofuran rings possessing no-adjacent in chain. Numbers of tetrahydrofuran rings in chain can be decisive in acetogenin larvae toxicity where bis-tetrahydrofuran can be twice stronger than mono-tetrahydrofuran (Blessing et al. 2010). Some published studies have shown the position of tetrahydrofuran rings in chain can be also related to acetogenin toxicity (Colom et al. 2007; He et al. 1997). Acetogenins are potent inhibitors of complex I (NADH: ubiquinone oxidoreductase) of the mitochondrial electron-transport system and of the coenzyme NADH in the cell membranes of target arthropods (Lewis et al. 1993). However, based on more recent studies, the variability of biological effects and the different functional groups in the form of compound chains indicate that inhibition of the mitochondrial complex is likely not the only mode of action of acetogenins (Blessing et al. 2012; Ribeiro et al. 2013).

The toxicity of six mono-tetrahydrofuran acetogenins isolated from *A. montana* on *S. frugiperda* was evaluated with exposure in artificial diet (100 ppm) and observed 100% mortality (larval and pupal stages) in some treatments. The authors identified annonacin, cis-annonacin-10-one and gigantetronenin, which cause high larval mortality (50%), as the most promising acetogenins (Blessing et al. 2010). Acetogenin bis-tetrahydrofuran rolliniastatin-1 from ESAM, that was tested at concentration of 84 ppm, causes 90 and 100% mortality of *S. frugiperda* larvae after seven days of exposure in treated artificial diet (Ansante et al. 2015). In the present study, after 5 days of exposure, total mortality of *H. armigera* larvae occurred at a concentration of 41.55 ppm (10% LC_{50}), with these larvae showing greater susceptibility than those of *S. frugiperda*.

H. armigera was recently introduced into Brazil and synthetic insecticides are registered for temporary use, and therefore, the use of botanical insecticides provides an alternative control measure for implementation within an integrated pest management (IPM) program, particularly for organic systems of vegetable crops such as tomato. Although the development of formulations involving products of botanical origin still faces challenges, such as increasing the stability, the use of *A. mucosa* ethanolic seed extract (ESAM) appears to be promising because the product is easy to obtain (maceration in ethanol) at a low cost and is harmless to plants (no phytotoxicity). The efficacy of this extract was comparable with that of the acetogenin-based commercial bioinsecticide and the flubendiamide-based commercial insecticide in semi-field trial, with the latter on record for emergency use in Brazil to control this pest. These results confirmed the potential of *A. mucosa* extracts as an alternative control measure for this important agricultural pest, which corroborates previously obtained results (Ribeiro et al. 2013, 2014a, b, c; Ansante et al. 2015) and expands the spectrum of action for the derivatives from this species of Annonaceae native to Brazil.

Authors' contribution

ELLB, LPR, CMS and JDV conceived and designed the research. CMS, RM and IFS conducted the experiments. JBF and KUB performed the chemical analysis. LPR analyzed the data. CMS, ELLB and LPR wrote the manuscript.

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Compliance with ethical standards

Conflict of interest All authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human or animal performed by any of the authors.

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