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PEST MANAGEMENT

Acetogenin-Based Formulated Bioinsecticides on *Anastrepha fraterculus*: Toxicity and Potential Use in Insecticidal Toxic Baits

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Keywords
Annonaceae, botanical insecticide, push-pull, fruit fly, integrated pest management, attract-and-kill

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Abstract
The present study evaluated the lethal toxicity and oviposition deterrence of ethanolic extracts of *Annona mucosa* Jacq., *Annona muricata* L., and *Annona sylvatica* A. St.-Hil on *Anastrepha fraterculus* (Wiedemann) (Diptera: Tephritidae) compared with those of a limonoid-based bioinsecticide (Azamax™ 1.2 EC—azadiractin +3-tigloyl-azadiractol) and a synthetic spinosyn-based insecticide (Delegate™ 250 WG—spinetoram). In addition, the efficacy of the selected toxic bait formulations was evaluated by mixing them with food attractants (Anamed™, 3% Biofruit and 7% sugarcane molasses). In the topical application and ingestion bioassays (2000 mg L^{-1}), the aqueous emulsion of the *A. mucosa* extract caused greater than 80% mortality of *A. fraterculus* adults in a similar manner to the spinosyn-based synthetic insecticide. Concentration-response curves were performed for the most promising treatments and showed an activity level dependent on the mode of contamination, exposure time, and applied concentration. In bioassays with and without choice, the *A. mucosa* (77%), *A. muricata* (51%), *A. sylvatica* (60%), Azamax™ (74%), and Delegate™ 250 WG (100%) significantly reduced the number of punctures and galleries in grape berries. In combination with the food attractants Anamed™, 3% Biofruit, and 7% sugarcane molasses, the emulsion of the *A. mucosa* extract had a residual effect similar to that of the spinetoram insecticide, with a mortality rate of over 80% of *A. fraterculus* adults up to 14 days after application (DAA) in the absence of rain. Thus, acetogenin-rich formulations, especially from *A. mucosa* seeds, are useful alternatives for the integrated management of *A. fraterculus* in agricultural orchards.

Introduction

One of the biggest challenges for the commercial cultivation of fresh fruit in the world is the presence of fruit flies in the producing regions (Malavasi 2000). In Brazil, the South American fruit fly *Anastrepha fraterculus* (Wiedemann) (Diptera: Tephritidae) is the most abundant pest species in orchards (Bortoli *et al* 2016). This species is characterized by its great capacity for multiplication and polyphagia, with development already reported in more than 100 host species, both native and cultivated (Lopes *et al* 2015). The direct damage is caused when females insert their ovipositor into the fruits and by larvae that develop inside them, causing rot and early loss of fruits (Botton *et al* 2016). In addition, the oviposition punctures in fruits favor the entry of pathogens (indirect damage), which accelerate fruit rot (Machota *et al* 2013).

The management of *A. fraterculus* populations is predominantly performed with synthetic chemicals through full-area

spraying, especially with organophosphorous, pyrethroid, or spinosyn insecticides and, more recently, with the use of toxic baits (attract-kill strategy) consisting of a mixture of the synthetic product (lethal agent) and food attractants (Borges *et al* 2015, Härter *et al* 2015, Botton *et al* 2016, Baronio *et al* 2018, Raga & Galdino 2018). However, due to growing concerns about the effects of synthetic products on human health and the environment (Biondi *et al* 2013, Böckmann *et al* 2014), some broad spectrum insecticides have been withdrawn from the market or banned for use in integrated fruit production (e.g., malathion) in Brazil (Botton *et al* 2016). In view of this, the development of new approaches and alternative products for the management of fruit flies, especially those more environmentally sustainable for the control of *A. fraterculus*, constitute an imminent necessity for the fruit production systems in Brazil and elsewhere (Navarro-Llopis *et al* 2011).

In this context, formulated products based on spinosyns, metabolites naturally obtained through the fermentation of the bacterium *Saccharopolyspora spinosa*, have been widely used (Markussen & Kristensen 2011, Galm & Sparks 2015) and have high toxicity on various species of fruit flies (Stark *et al* 2004, Flores *et al* 2011, Baronio *et al* 2018, Schutze *et al* 2018). In addition, the use of natural products is an important strategy for integrated pest management (IPM) programs in tropical and subtropical fruit production (Pretty *et al* 2018, Amoabeng *et al* 2019). Currently, several species of tropical plants have been identified as potential sources of compounds that can be used in the elaboration of botanical insecticides, especially species belonging to the families Annonaceae, Asteraceae, Canellaceae, Lamiacae, Malvaceae, Meliaceae, and Rutaceae (Isman & Seffrin 2014, Moghadamtousi *et al* 2015). In the Neotropical region, the Annonaceae family is considered one of the most promising (Ribeiro *et al* 2016) due mainly to the presence of acetogenins, a class of compounds exclusively found in some genera of this botanical family and present in high concentration in seeds, especially in those of species of the *Annona* genus (Alali *et al* 1999, Isman 2006, Isman & Grieneisen 2014, Ribeiro *et al* 2016). Due to the diverse structure, acetogenins cause potent lethal effect on pest species with different eating habits, as well as sublethal effects regarding feeding behavior and oviposition (Isman & Seffrin 2014, Ribeiro *et al* 2015, Bernardi *et al* 2017). However, thus far, neither the effects of acetonin-rich Annonaceae derivatives on fruit flies nor the insertion of these compounds in attract-kill strategies has been evaluated. Thus, the development of new fruit fly management alternatives that meet the requirements of organic production is one of the main needs of fruit production chains, and botanical insecticides are important tools in this context within the precepts of IPM (Pretty *et al* 2018, Amoabeng *et al* 2019).

Considering the previously described, the present study aimed to evaluate the lethal toxicity of formulations based on acetogenin-rich ethanolic extracts obtained from seeds of preselected *Annona* species (*Annona mucosa* Jacq., *Annona muricata* L., and *Annona sylvatica* A. St.-Hil) on *A. fraterculus* adults. In addition, the effect of these extracts formulated in admixtures with a food attractant for push-pull formulation was also evaluated, a strategy widely used for the management of fruit fly complexes in orchards. Finally, the effect of these derivatives on the oviposition behavior of *A. fraterculus* was evaluated in grape berries.

Materials and Methods

Botanical derivatives: sources, method of preparation and formulation procedure

Information on the *Annona* species used in this study is detailed in Table 1. Voucher specimens, 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 from School of Agriculture "Luiz de Queiroz"/ University of São Paulo, in Piracicaba, SP, under the registered numbers 120985 (*A. mucosa*), 121205 (*A. sylvatica*), and 121892 (*A. muricata*).

For the preparation of crude ethanolic extracts, the seeds collected from ripe fruits were dried in an oven with forced air circulation (Prolab MBSSDC64L model) at 38°C for 48 h and ground in a knife mill (Solab - GRINDOMIX GM 200 model) until a fine powder was obtained. The powders were placed in glass containers, sealed, and stored at approximately − 10°C until use.

Organic extracts were obtained by cold maceration using analytical-grade ethanol as the solvent (5:1, v v^{-1}). For this, the seed powder of the respective species was added to the solvent, stirred for 10 min, and incubated at rest for 3 days. After this time, the solution was filtered and the remaining solids were subjected again to the same solvent at the same ratio, repeating this process three times. The remaining solvent in the sample was removed by rotary evaporation at 50°C and 600 $mmHg^{-1}$. After complete evaporation of the solvent in an airflow chamber, the extraction yield for each species was determined. For the preparation of the formulations (aqueous emulsions), the ethanolic extracts of the seeds were solubilized in acetone:methanol (1:1 v v^{-1}) a 100 g L^{-1}, with subsequent addition of emulsifier Tween® 80 in the concentration of 10 g L^{-1}.

Table 1 Treatments evaluated for the management of *Anastrepha fraterculus*

Treatments	Description	Discriminatory concentration tested[a]	Origin/manufacturer
EES Annona mucosa	Aqueous emulsion of ethanolic seed-extract of *Annona mucosa* Jacq. (pre-commercial)	2000	Laboratory extraction and formulation
EES Annona muricata	Aqueous emulsion of ethanolic seed-extract of *Annona muricata* L. (pre-commercial)	2000	Laboratory extraction and formulation
EES Annona sylvatica	Aqueous emulsion of ethanolic seed-extract of *Annona sylvatica* A. St.-Hil. (pre-commercial)	2000	Laboratory extraction and formulation
Azamax™ 12 EC	Limonoid-based biopesticide [azadirachtin (6220.15 mg L^{-1}) + 3-tigloylazadirachtol (2596 mg l^{-1})] extracted and purified from *Azadirachta indica* L. seeds	3 ml L^{-1} (recommended by the manufacturer)	UPL Brasil (Campinas, SP, Brazil)
Delegate 250WG™	Spinetoram (250 g kg^{-1})	300[b]	Corteva Agriscience™, São Paulo, SP, Brazil

[a] Concentration: milligrams of extract or commercial product per liter of water

[b] 75 mg a.i. L^{-1} of water

ESE, formulated ethanolic seed extract

Insects

The bioassays were performed using *A. fraterculus* adults from a population kept in the laboratory for approximately 20 generations, free of insecticide selection pressure. To maintain the population, insects were kept on an artificial diet proposed by Nunes *et al* (2013) under controlled conditions (temperature 25 ± 1°C; relative humidity 70 ± 5%; and photophase 12 h).

Bioassays

All bioassays were performed under controlled conditions under a completely randomized design. The treatments and discriminatory concentrations used are detailed in Table 1. A spinosyn-based formulation (Delegate ™ 250 WG, 250 g active ingredient (a.i.) kg^{-1}) (Corteva Agriscience, Sao Paulo, SP, Brazil) and limonoid-based bioinsecticide (Azamax ™ 1.2 EC, azadiractin +3-tigloyl-azadiractol, 12 g a.i. L^{-1}) (UPL Brazil, Campinas, SP, Brazil) were used as positive controls (Table 1). As a negative control, the solvents used in the solubilization of the respective treatments were used.

Initial screening (ingestion and topical application bioassays)

To assess the lethal toxicity of the formulations prepared from the derivatives obtained, an initial screening was performed using the discriminatory concentration of 2000 mg L^{-1} on *A. fraterculus* adults (Table 1). For this purpose, bioassays of ingestion and topical application were used.

Ingestion bioassay

For this mode of contamination, the bioinsecticides (Table 1) were mixed in different food attractants: Anamed™ (40% SPLAT ™ + 24.2% of food containing fruit extracts and phytostimulant (Isca Tecnologias Ltda., Ijuí, RS, Brazil)), Biofruit at 3% (hydrolyzed protein (BioControle Métodos de Controle de Pragas Ltda., Indaiatuba, SP, Brazil)), and 7% sugarcane molasses (homemade preparation). As a negative control, food attractants were mixed in water. Therefore, the insects were separated into groups (sample units) of 20 couples (8 days old) and placed in cages made of transparent plastic Petri dishes (1 L) that were overturned onto plastic Petri dishes (25 cm diameter) and sealed on top (bottom of the container) with voile mesh for ventilation. For this bioassay, adults of *A. fraterculus* up to 8 days old were deprived of food for 12 h prior to the provision of the toxic bait formulations, as suggested by Baronio *et al* (2019). After the preparation of the toxic bait dilutions (bioinsecticide + food attractant), the products were offered to the insects by capillary hydrophilic cotton rolls in 10-mL glass bottles. After 24 h, the toxic baits were removed and the adults were fed an artificial diet and distilled water until the end of the evaluation period (Nunes *et al* 2013). Mortality was assessed daily for 5 days. Insects were considered dead when no movement occurred after touching them with a thin brush. For each treatment, four replicates were used, each composed of 20 adults ($n = 80$).

Topical application bioassay

Groups of 10 couples of *A. fraterculus* (8 days old) were separated and placed in clear glass tubes (2.5 cm diameter × 8 cm long) sealed with cotton plugs. Subsequently, the insects were sedated in a freezer (~ − 10°C) for 30 s and placed on a glass Petri dishes (9 cm diameter) lined with filter paper. The insects were then sprayed using a Potter spray tower (Burkard Scientific, Uxbridge, UK), applying 1 mL of solution per sample unit at a working pressure of 7 lb. in^{-2},

resulting in an average residue deposition of 3 mg cm^{-2}. Then, the insects were placed in transparent plastic cages (1000 mL) sealed with a vented lid (a voile mesh-sealed opening in the container lid) as described previously. The adults were fed an artificial diet and distilled water in the same way described previously until the end of the evaluation period. Again, mortality was assessed daily for 5 days. As a negative control was only used water. For each treatment, 10 replicates were performed, with 10 adults per replicate ($n = 100$). The corrected mortality was calculated using the formula of Abbott (1925).

Concentration-response curves

Based on the initial screening, the most promising treatments were selected and subjected to a new bioassay to estimate the concentration required to kill 50% and 90% of exposed flies (LC_{50} and LC_{90}, respectively). Seven concentrations were tested (range 125–4000 mg L^{-1} for the formulated extract of *A. mucosa* seeds and 10–100 mg L^{-1} for spinetoram). Exposure and application modes, as well as mortality criteria and exposure times, were the same as those used for the initial screening (ingestion and topical application bioassays). In the intake bioassays, four replicates were performed; each replicate composed of 20 adults ($n = 80$). In the bioassay of topical application, 10 replicates were performed with 10 adults exposed in each of them ($n = 100$).

Oviposition deterrence evaluation

To evaluate the effect of the treatments (Table 1) on the oviposition behavior of *A. fraterculus*, adults were submitted to bioassays with and without choice using mature 'Italy' grape berries (intact and free from insecticide contamination) as an oviposition substrate.

In the bioassay with no choice, the grape berries were dipped in test solutions (Table 1) diluted in water for 5 s and then dried on filter paper for 3 h. Then, the grape berries were placed inside cages made by transparent plastic containers (1000 mL) inverted on Petri dishes (8 cm in diameter) (one per cage). Subsequently, two pairs of *A. fraterculus* (13 days old) couples were placed in the container. The insects were fed an artificial diet + distilled water.

The bioassay with choice was conducted in plastic cages under the same conditions as the bioassay with no choice. However, two intact grape berries (one treated berry (immersed in the test solution of the treatments as described previously) and untreated (one berry without contact with the treatments)) were placed in each cage. Subsequently, each cage was infested with two pairs of *A. fraterculus* (13 days old).

For both bioassays, after 24 h, the adults were removed and the grape berries were individually placed inside plastic containers (50 mL) on a vermiculite layer (1 cm), sealed with Parafilm™ and placed in a room (25 ± 1°C and 70 ± 5% relative humidity with a 12 h photophase). After 5 days, the number of "punctures" was counted, that is, oviposition marks caused by female *A. fraterculus*, and the presence of galleries, as well as the evaluation of possible larval hatching. The number of punctures and galleries was counted using a stereoscopic microscope (40 times). In both test modalities, the experimental design was completely randomized with 50 berries (replications)/treatment.

*Effectiveness of insecticidal toxic bait formulations on **A. fraterculus** adults*

To evaluate the residual effect of toxic baits in the absence of rainfall on medfly mortality, 2-year-old *Citrus sinensis* L. (Rutaceae) seedlings, 1.5 m in height, were grown in pots inside a greenhouse (25 ± 2°C, 70 ± 10% relative humidity, and a 12-h photoperiod). The toxic baits were applied on the leaves, which were collected after bait drying on the day of application (2 h after application, 0 days) and at 7, 14, 21, and 30 days after application of the treatments (DAA). The samples were transported to the laboratory and supplied to five males and five females of *A. fraterculus* adults 5 to 8 days old that were food deprived for 12 h and in separate cages. Plastic containers with cotton wool soaked in distilled water were offered inside 5-mL plastic containers during the evaluation period. The cage top was lined with a white "voile" type fabric to facilitate the viewing and counting of dead insects. The number of dead individuals in each treatment was evaluated 120 h after the exposure to treatment (HAET). The mortality was evaluated by counting the number of insects that did not show any reaction to the touch of a fine-tipped brush. All experiments were conducted in a completely randomized design with 10 replicates with 10 adult fruit flies each. The efficacy of each treatment was calculated using the formula of Abbott (1925).

Statistical analysis

For the analysis of the studied variables, generalized linear models (GLM) belonging to the exponential family of distributions were used (Nelder & Wedderburn 1972). The quality of the fit was verified by means of the half-normal probability envelope with a simulation envelope (Hinde & Demétrio 1998). When significant differences between treatments were detected, multiple comparisons (Tukey post hoc test, $p < 0.05$) were performed using the multcomp package glht function, with adjustments of the p values. To compare the mean treatments in the oviposition deterrence bioassay (with choice), Student's t test was used. All analyses were performed with "R" statistical software (R Development Core Team 2012). A binomial model with a complementary log-log binding

function (gompit model) was used to estimate lethal concentrations (CL_{50} and CL_{90}) using the Probit package of the SAS version 9.2 statistical software package (SAS Institute 2011).

Results

Lethal toxicity and concentration-response curves of the active treatments

In the bioassay of topical application, the aqueous emulsion of the ethanolic extract of *A. mucosa* seeds caused a mortality rate of approximately 80% (Fig 1), not differing from that of the spinosyn-based positive control (250 g kg^{-1}) (Fig 1) ($F_{3, 24} = 74.69$, $p < 0.0001$). In contrast, the aqueous emulsions of the ethanolic extracts of *A. muricata* and *A. sylvatica* seeds caused mortality of approximately 60% (Fig 1). In the ingestion bioassay, 120 h after exposure of insects to treatment, the attractive food had no influence on mortality of *A. fraterculus* adults (Table 2). The seed extract emulsions mixed with the food attractants, Anamed™ ($F_{3, 24} = 40.61$, $p < 0.0001$), 3% Biofruit ($F_{3, 24} = 71.93$, $p < 0.0001$), and 7% sugarcane molasses ($F_{3, 24} = 42.73$, $p < 0.0001$), caused mortality rates higher than 80%, which were similar to those of the insecticide spinetoram in mixtures with the attractants Anamed™ (88.0% mortality), 3% Biofruit (86.0% mortality), and 7% sugarcane molasses (94.0% mortality) (Table 2).

Based on the adjusted concentration-response curves, in topically applied bioassay (120 h exposure), the estimated LC_{50} for the *A. mucosa* seed extract emulsion was significantly lower ($LC_{50} = 520.71$ mg L^{-1}) than that observed with the spinosyn-based insecticide ($LC_{50} = 41.96$ mg L^{-1}) (Table 3), as similarly observed for LC_{90} values, *A. mucosa* seed extract (5013.12 mg L^{-1}) and spinetoram insecticide (LC_{90} 93.62 mg L^{-1}) (Table 3). In ingestion bioassay, the lethal toxicity of the *A. mucosa* extract-based formulation was lower ($LC_{50} = 728.36$ mg L^{-1}) than that of spinetoram ($LC_{50} = 35.14$ mg L^{-1}) after 120 h of exposure (Table 3). Considering the adjusted LC_{90} values for both treatments (3212.06 mg L^{-1} and 89.24 mg L^{-1}, respectively), the same trend was observed (Table 3).

Oviposition deterrence

In the bioassays with no choice, residues from all treatments significantly reduced the number of punctures per grape berry ($F_{6, 693} = 224.1$, $p < 0.0001$) caused by females of *A. fraterculus* (Fig 2A). Similarly, a significant reduction in the number of punctures in the choice bioassay was observed in all evaluated treatments compared with that of the negative control (water) (Fig 2B). The smaller number of punctures resulted, consequently, in a lower number of galleries per grape berry caused by *A. fraterculus* larvae, including those observed after treatment with the emulsions of the *A. mucosa* seed extract (0.60 galleries per berry), *A. muricata* (0.88), and *A. sylvatica* (0.72), the limonoid-based formulation (0.47) and spinetoram (total inhibition), which all provided a significant reduction ($F_{6, 693} = 88.79$,

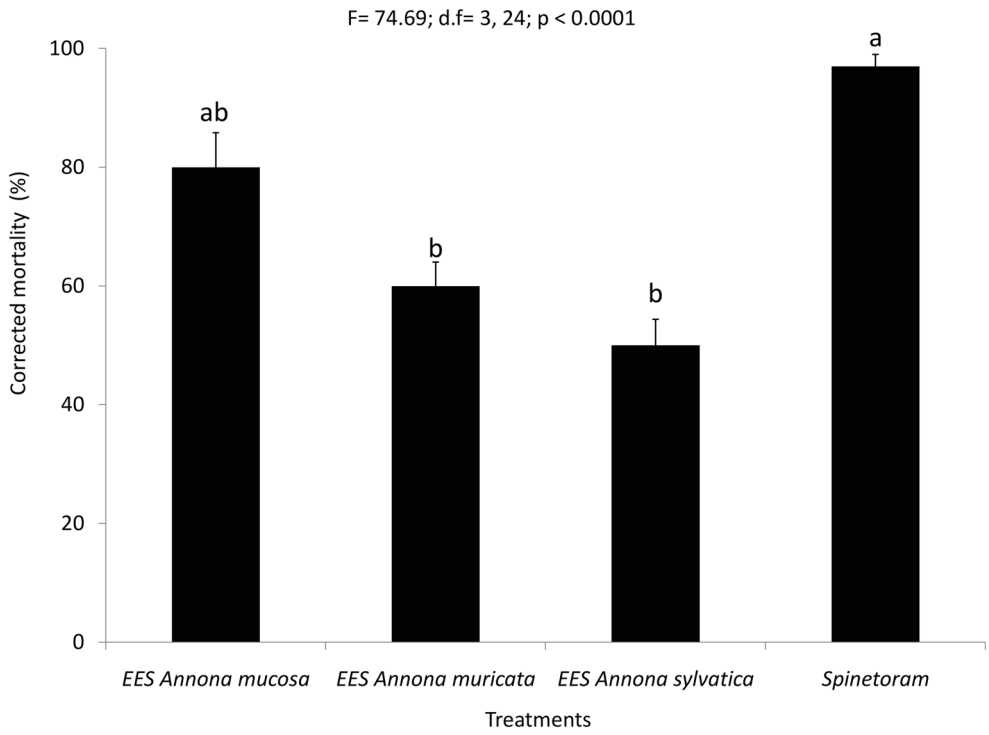

Fig 1 Mortality (%) (means ± SE) of *Anastrepha fraterculus* at 120 h after exposure in laboratory bioassays by means of topical application.

Table 2 Corrected mortality percentage (mean ± SE) of *Anastrepha fraterculus* after 120 h of exposure to formulations based on Annonaceae derivatives and spinosyns (spinetoram) in admixture with different food and water attractants in a bioassay

Treatments	Food attractants			
	Anamed™	3% Biofruit	7% Sugarcane molasses	Control (water)
EES *A. mucosa*	100.0 ± 0.00 a	94.0 ± 2.45 a	84.0 ± 6.00 a	98.0 ± 2.00 a
EES *A. muricata*	64.0 ± 8.72 b	42.0 ± 4.90 b	30.0 ± 4.48 b	42.0 ± 5.83 b
EES *A. sylvatica*	54.0 ± 6.00 b	14.0 ± 2.45 c	26.0 ± 5.10 b	46.0 ± 2.46 b
Spinetoram	88.0 ± 4.90 a	86.0 ± 4.00 a	94.0 ± 2.45 a	94.0 ± 4.00 a
F	40.61	71.93	42.78	44.78
df	3, 24	3, 24	3, 24	3, 24
p values	< 0.0001	< 0.0001	< 0.0001	< 0.0001

Means within a column followed by the same letter do not differ significantly (GLM with a quasi-binomial distribution followed by Tukey post hoc test: $p < 0.05$)

EES, formulated ethanolic seed extract

Mixtures with each food attractant were tested separately in independent bioassays

$p < 0.0001$) when compared with that of the negative control (1.77 galleries per berry) (Table 4).

*Effectiveness of selected treatments on toxic bait formulation on **A. fraterculus** adults*

In toxic bait formulations, emulsion of the *A. mucosa* seed extract in combination with attractants food (Anamed™, 3% Biofruit and 7% sugarcane molasses) provided high mortality of *A. fraterculus* adults after 0, 7, 14, 21, and 30 DAA when compared with that of toxic baits formulated with the spinetoram insecticide in the absence of rain (Fig 3A, B and C). In addition, until 14 DAA, the mortality provided by the emulsion of *A. mucosa* seed extract was greater than 80% (Fig 3A, B and C). In assessing the toxic baits over time at 14, 21, and 30 DAA, there was a significant reduction ($p < 0.05$) in mortality of adults of *A. fraterculus* in all toxic bait formulations evaluated (*A. mucosa* or spinetoram in combination with attractive food Anamed™, 3% Biofruit and 7% sugarcane molasses) (Fig 3A, B and C).

Discussion

The lethal and sublethal toxicity of acetogenin-rich Annonaceae derivatives has been widely characterized for different species of pest arthropods with different eating habits, including *Sitophilus zeamais* Motschulsky (Coleoptera: Curculionidae) (Ribeiro *et al* 2013; Ribeiro & Vendramim 2017), *Panonychus citri* (McGregor) (Prostigmata: Tetranychidae) (Ribeiro *et al* 2014a), *Trichoplusia ni* Hübner (Lepidoptera: Noctuidae) and *Myzus persicae* (Sulzer) (Hemiptera: Aphididae) (Ribeiro *et al* 2014b), *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae) (Ansante *et al* 2015), *Helicoverpa armigera* (Lepidoptera: Noctuidae) (Souza *et al* 2019), *Drosophila suzukii* (Matsumura) (Diptera: Drosophilidae) (Bernardi *et al* 2017), and *Zaprionus indianus* Gupta (Diptera: Drosophilidae) (Geisler *et al* 2019). However, for the first time, this study reports the lethal and sublethal toxicity of acetogenin-rich derivatives on a fruit fly species (*A. fraterculus*), the main pest species of tropical and temperate fruits, as well as the possibility of using these derivatives in toxic bait formulations.

Table 3 Estimate of the LC_{50} and LC_{90} (in mg L^{-1}) according the confidence interval of a formulated ethanolic extract from *Annona mucosa* seeds (Annonaceae) and a spinosyn-based synthetic insecticide (spinetoram) on *Anastrepha fraterculus* adults in topical application and ingestion bioassays

Treatments	*n*	Slope ± SE	LC_{50} (CI 95%)[a]	LC_{90} (CI 95%)[b]	χ^{2c}	df[d]
Topical application bioassay						
EES *Annona mucosa*	580	2.15 ± 0.27	520.71 (270.47–995.70)	5013.12 (3763.84 – 7398.12)	9.75	5
Spinetoram	720	3.84 ± 1.14	41.96 (17.14–52.74)	93.62 (52.29–114.73)	8.70	5
Ingestion bioassay						
EES *Annona mucosa*	580	2.78 ± 0.21	728.36 (450.75–994.73)	3212.06 (2763.84 – 4398.12)	7.60	5
Spinetoram	720	2.84 ± 0.31	35.14 (27.14–42.74)	89.24 (62.29–104.53)	8.70	5

[a,b] LC_{50} and LC_{90}: concentrations (mg L^{-1}) required to kill 50 or 90% of the adults of *A. fraterculus*, respectively

CI, confidence interval at 95%; [c] χ^2, Pearson's chi-square value; [d] *df*, degrees of freedom; *EES*, formulated ethanolic seed extract

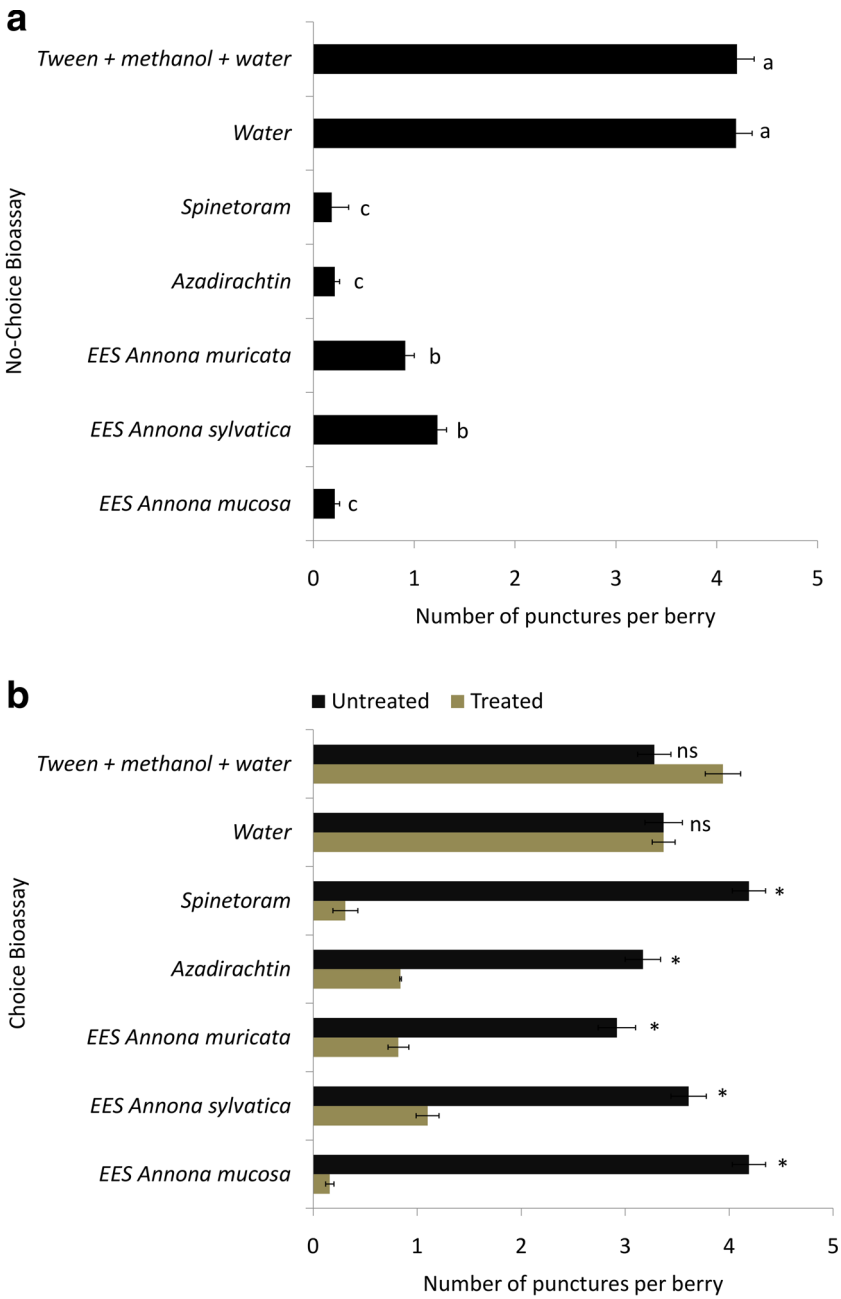

Fig 2 Means (± SE) of number of punctures caused by *Anastrepha fraterculus* females in grapes berries treated with different (bio) insecticides: (A) bioassay no choice (means followed by distinct letters in bars indicate significant differences between treatments (GLM with quasi-Poisson distribution followed by Tukey's post hoc test, $p < 0.05$)). (B) Choice-based bioassay (asterisks indicate significant differences between treatments according to Student's *t* test ($p < 0.05$) and "ns" not significant according to Student's *t* test ($p < 0.05$))

The high toxicity of the aqueous emulsion of the *A. mucosa* seed extract on *A. fraterculus* adults is possibly related to the presence of bis-tetrahydrofuran acetogenin rollinistatin-1, a major compound found in this derivative (Ansante *et al* 2015). According to Tormo *et al* (1999), acetogenins act to inhibit mitochondrial complex I (NADH: ubiquinone oxidoreductase) of the mitochondrial electron transport system, inducing programmed cell death (apoptosis) by ATP production deprivation. In addition to acetogenins, other secondary compounds can be found in Annonaceae seed extracts, including alkaloids (Ribeiro *et al* 2013, 2014a), which may interact synergistically for the observed global bioactivity. Although there are no reports of evolution of *A. fraterculus* resistance to insecticides in Brazil (Raga & Galdino 2018), the availability of new insecticidal molecules, mainly of natural origin, may be of paramount importance to manage the pest since the products used for the control of *A. fraterculus* in commercial orchards (up to six applications per crop) have been synthetic insecticides, pyrethroid,s and spinosyns (Scoz *et al* 2004, Härter *et al* 2010, Borges *et al* 2015, Botton *et al* 2016, Raga & Galdino 2018). In addition, the availability of products with different modes of action on fruit flies will contribute to insecticide rotation and/or slow the evolution of fruit fly

Table 4 Number of galleries (mean ± SE) caused by *Anastrepha fraterculus* larvae in grape berries treated by immersion in different treatments

Treatments	Number of galleries
EES *Annona mucosa*	0.60 ± 0.06 bc
EES *Annona muricata*	0.88 ± 0.06 b
EES *Annona sylvatica*	0.72 ± 0.05 bc
Azadirachtin +3-tigloyl-azadirachtol	0.47 ± 0.06 c
Spinetoram	0.00 ± 0.00 c
Water	1.77 ± 0.10 a
Water + methanol + Tween	1.84 ± 0.11 a
F	88.791
df	6, 693
p values	< 0.0001

Means followed by different letters on the columns indicate significant differences between treatments (GLM with quasi-Poisson distribution followed by post hoc Tukey test, $p < 0.05$)

EES, formulated ethanolic seed extract

resistance (Couso-Ferrer *et al* 2011; Vontas *et al* 2011; Arouri *et al* 2015; Baronio *et al* 2019).

Although the ethanolic extracts of *A. muricata* and *A. sylvatica* seeds did not show high toxicity in adults of *A. fraterculus*, together with the limonoid-based insecticide, such bioinsecticides provided a reduction in the puncture number and, consequently, the number of galleries in grape berries. The oviposition deterrent effect can be a useful component to reduce the pest population in the orchards, reducing the oviposition in the fruits and, consequently, the entry of pathogens that accelerate the deterioration, reducing the viability of the product for the consumer (Machota Júnior *et al* 2013).

In this study, a high adult mortality rate of *A. fraterculus* was found when exposed to toxic bait formulations containing the aqueous emulsion of an *A. mucosa* seed extract in mixtures with the food attractants Anamed™, 3% Biofruit, and sugarcane molasses. Thus, formulations based on this botanical derivative can be used in push-pull strategies, and their activity level is comparable to that of a spinosyn-based insecticide, a product widely used for this strategy in Brazil (Raga and Sato 2005, Härter *et al* 2015, Baronio *et al* 2018, Raga & Galdino 2018, Schutze *et al* 2018) and other regions of the world (Prokopy *et al* 2004, Revis *et al* 2004, Mangan 2009, Flores *et al* 2011, Yee & Alston 2016). Although the aqueous emulsion of the *A. mucosa* extract showed a residual effect and high toxicity (mortality up to 80% at 14 DAA) to *A. fraterculus* adults when associated with different food attractants in the absence of rain, one of the limitations in the use of these formulations of toxic baits is the low persistence in the field, especially in regions where frequent rainfall occurs, such as subtropical regions (Prokopy *et al* 2003,

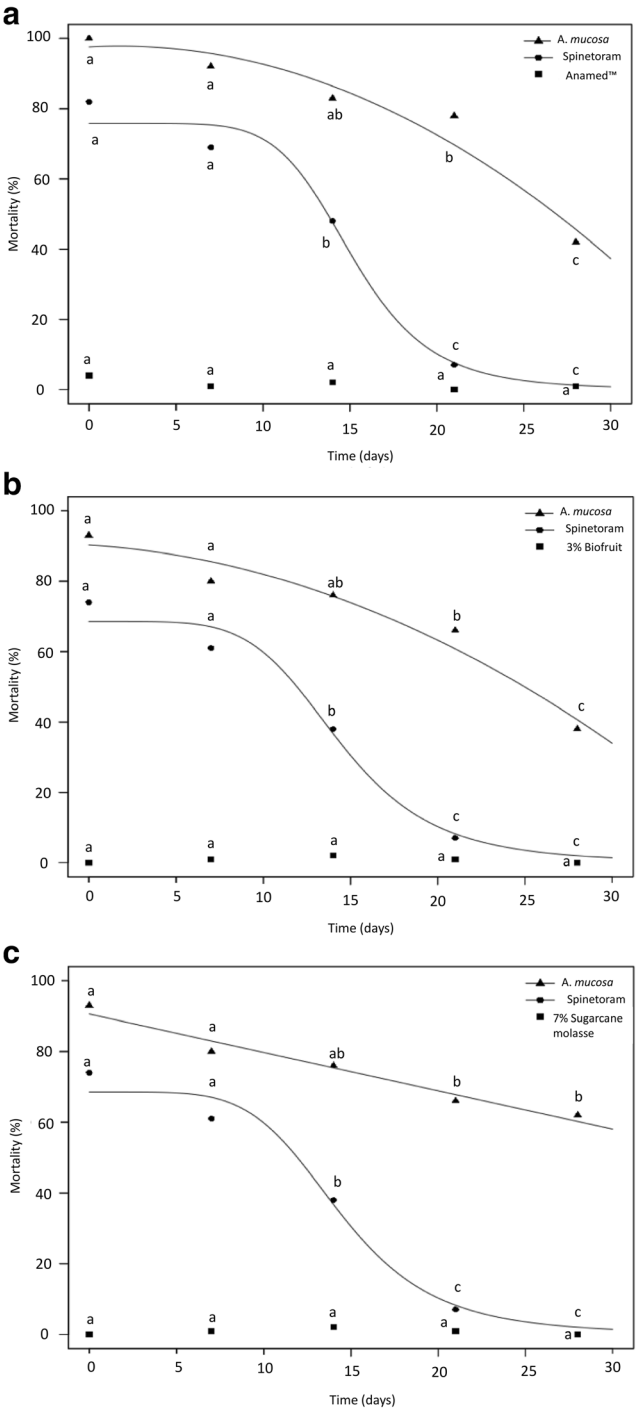

Fig 3 Mortality (%) of *Anastrepha fraterculus* after 24 h of exposure to toxic bait residues with different food attractants at 0, 7, 14, 21, and 30 days after application (DAA), in absence of rain. (A) Anamed™; (B) 3% Biofruit, and (C) 7% sugarcane molasses

Revis *et al* 2004). In view of this, in future studies, the residual power of the formulations (*A. mucosa* aqueous emulsion + food attractants) will be investigated in the presence of rain, as verified with toxic spinosyn-based baits (Revis *et al* 2004, Flores *et al* 2011, Borges *et al* 2015, Härter *et al* 2015, Baronio *et al* 2019).

Despite the availability of some commercial Annonaceae-based bioinsecticides in Middle Eastern countries (i.e., Anoson®, BioRakshak®, and AnonaCin®), the greatest diversity of these plant species is found in the Neotropical region, many of them being cultivated on a commercial scale in Brazil, and the seeds are considered an agro-industrial residue in fruit processing industries (Isman & Seffrin 2014, Ribeiro *et al* 2014a). Therefore, these residues are a low-cost and abundantly available source for bioinsecticide production, transforming an environmental problem into an ecofriendly solution for agriculture. In this study, the formulation with the ethanolic extract of *A. mucosa* seeds showed promising bioactivity on *A. fraterculus*, with toxic effects comparable to those of a spinosyn-based commercial insecticide and superior to those of a commercial limonoid-based botanical insecticide. Furthermore, the ethanolic extract of *A. mucosa* seeds has been shown to have a superior residual effect compared with that of a synthetic insecticide when formulating toxic baits, which may be a useful component in organic production systems or to reduce the pressure of *A. fraterculus* population selection by organosynthetic insecticides.

Author contribution statement DB, DEN, and LdPR planed and designed research; PS, DdCO, LNM, and FCSG conducted experiments; DB, MR, LdPR, and DEN conducted statistical analysis and wrote the manuscript. All authors read and approved the manuscript.

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