Botanical insecticides and extracts and their lethal and sublethal effects on *Anastrepha obliqua* (Macquart) (Diptera: Tephritidae) and *Diachasmimorpha longicaudata* (Ashmead) (Hymenoptera: Braconidae)

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Abstract

Knowledge of the action of chemical and botanical insecticides on pests and natural enemies is essential for improving integrated management programs of fruit flies. The aimed evaluate the toxic and sublethal effects of botanical insecticides and extracts on *Anastrepha obliqua* and *Diachasmimorpha. longicaudata*. Botanical extracts and insecticides were applied to fruit flies or parasitoids, using a methanol extract of leaves of *Aspidosperma spruceanum* (MLAS), ethanol extract of bark of *Metrodorea maracasana* (EBMM), hexane extract of leaves of *Conchocarpus mastigophorus* (HLCM), ethanol extract of leaves of *C. mastigophorus* (ELCM), ethyl acetate extract of leaves of *Xylopia sericea* (ALXS), and oil of seeds of *Aleurites moluccana* (OSAM) in single concentration of 70 mg mL^{-1}, in addition to the insecticides Azamax® (5 mL L^{-1}) and Natuneem® (10 mL L^{-1}). Extract EBMM and the two insecticides were used for the lethal effect on the *A. obliqua* and parasitoid, and extract ELCM only in parasitoid. The extract EBMM was also evaluated on *A. obliqua* at concentrations of 60, 70, 80, 90, and 100 mg mL^{-1}. Sublethal effects was carried out with commercial products based on neem and water in adults *A. obliqua*. Azamax® and the extracts EBMM, ELCM, and HLCM were toxic to *A. obliqua*. Natuneem® and the extracts ELCM and EBMM were harmless to adults of the *D. longicaudata*. Azamax® was considered slightly harmful to the parasitoid. The extract EBMM was harmless to adults or larvae and moderately harmful in the double application. Extract EBMM has the potential for bioprospecting, aiming at tephritid management.

Introduction

Brazil has the largest plant biodiversity in the world, with more than 46,000 cataloged species and many others not yet identified (BFG 2018). These species are distributed in the Amazon, Atlantic Forest, Caatinga, Cerrado, Pampas, and Pantanal biomes (BFG 2015). The Caatinga biome, with a semi-arid climate, has a very diversified biological heritage, being the only biome located entirely within the Brazilian territory and corresponding to approximately 10% of the country (MMA 2011). Endemic plants in the Brazilian semi-arid region have shown promise for the discovery of new biologically active substances (Reis et al. 2010).

Fruit flies (Diptera: Tephritidae) are considered one of the main phytosanitary problems for fruit growing, as the larval stage of these insects develops inside the fruit, causing depreciation, early drop, and, consequently, unfeasibility for commercialization. The species *Ceratitis capitata* (Wiedemann 1824) stands out in Brazil, occurring in 94 species of native and exotic hosts (Zucchi and Moraes 2012), in addition to 128 species of the genus *Anastrepha* (Schiner 1868), among which the polyphagous species *Anastrepha fraterculus* (Wiedemann 1830) and *Anastrepha obliqua* (Macquart, 1835) (Zucchi and Moraes 2022) stand out due to their wide distribution and because they infest several plant species, although they prefer fruits from the families Anacardiaceae and Myrtaceae. However, these species have been found on new hosts, such as *A. obliqua* on forage cactus (Leite et al. 2017). Non-European species of Tephritidae, which in the case of Brazil include *A. fraterculus* and *A. obliqua*, began to have quarantine importance for the export of citrus and mango fruits from the publication of the European Union Directive 2019/523 of March 21, 2019 (União Europeia 2019).

Chemical control is the most used method for population suppression in the management of fruit flies (Diptera: Tephritidae) of economic importance for Brazil and other countries that produce or import fresh fruits. Although effective, the use of insecticides causes environmental and toxicological problems, in addition to biological imbalance by eliminating beneficial organisms present in the agroecosystem (Degrande et al. 2002). In this context, the use of biorational products such as plant extracts would be of paramount importance, as they affect pests, but have low toxicity to non-target organisms and natural enemies (Horowitz and Ishaaya 2004; Teixeira 2016). Bioprospection is an ally to the screening of biorational products, enabling formulations and modes of use of these natural products with potential in the management of agricultural and forestry pests (Pinto et al. 2002), and enabling the aggregation of value to this plant biodiversity.

Plant extracts have great potential for the development of strategies for suppression of fruit fly populations, as they have several advantages over synthetic insecticides, such as a low persistence in the environment stands out, contributing to food security, few possibilities of selection of resistant insect populations, reducing the environmental contamination, and compatibility with integrated pest management (IPM) programs (Barbosa et al. 2006).

Plant extracts and botanical insecticides can cause, in addition to the lethal toxic effects, several deleterious effects on insect pests, such as repellency, feeding inhibition, behavioral changes, adult sterilization, and morphogenetic and hormonal system changes, thus negatively affecting the biological development (Fernandes et al. 2017). These effects are considered sublethal, that is, they affect the biology, physiology, and behavior of insects, reducing the development, feeding, fecundity, longevity, sex ratio, mobility, oviposition, and prey/host search capacity (França et al. 2017). Lethal and sublethal effects are important when evaluating the selectivity of insecticides to natural enemies (Paiva et al. 2018).

Integrated pest management (IPM) programs in fruit farming have encouraged the use of various control methods and tactics, such as cultural and behavioral methods, varietal resistance, and, mainly, biological control. In Brazil, biological control of fruit flies is carried out mainly by parasitoids of the family Braconidae. The species *Diachasmimorpha longicaudata* (Ashmead, 1905) (Hymenoptera: Braconidae) was introduced in the 1990s by Embrapa Cassava and Fruits to reduce the population density of fruit flies and favor the increase in the population of natural enemies (Carvalho et al. 2000). In addition, *D. longicaudata* was chosen for introduction in Brazil due to its specificity for the family Tephritidae and its ease of rearing in the laboratory (Garcia and Ricalde 2013).

Substances with selective action in favor of natural enemies, mainly for the success of integrated pest management programs in agroecosystems, the maintenance of existing natural enemies, and/or the rearing and release of predators, pathogens, and parasitoids are essential in the search for new insect control agents (Torres et al. 2007).

Plants of the semi-arid region, endemic to the Brazilian flora, such as *Metrodorea maracasana* Kaastra (Rutaceae), found in the Northeast (Bahia) and Southeast (Espírito Santo), have been studied to control the fruit flies *C. capita* and *A. obliqua*, with promising results (Gomes 2014; Costa 2016). Neem (*Azadirachta indica* A. Juss) (Meliaceae) derivatives have also been used to control fruit flies, acting mainly in reducing oviposition (Machota Junior et al. 2013; Silva et al. 2015). Although important, studies on the toxicity of these plants to natural enemies are scarce, as they are more susceptible to insecticide compounds than pests. Thus, it is necessary to assess how these products affect the effectiveness of beneficial insects (Bueno et al. 2017). Studies have investigated the selectivity of insecticides to the parasitoid *D. longicaudata* (Ruiz et al. 2008; França et al. 2010; Alvarenga et al. 2012; Oliveira 2014), in general, with the product application only on the units of parasitism (groups of larvae), disregarding a probable sublethal effect on adult parasitoids that survived the application. Therefore, this study aimed to evaluate the toxic and sublethal effects of botanical insecticides and extracts on adults of *A. obliqua* and the parasitoid *D. longicaudata*.

Material and Methods

Origin of Anastrepha obliqua and Diachasmimorpha longicaudata

The studies were carried out at the Laboratory of Fruit Flies. Fruit flies of the species *A. obliqua* were obtained from EMBRAPA Cassava and Fruits and kept in the laboratory in a climate-controlled room, with a temperature of 25 ± 2°C and relative humidity of 70 ± 10%, in acrylic cages (30 × 30 × 30 cm) and fed daily a diet based on yeast extract + sugar (Silva-Neto et al. 2012) and water. Guava fruits of the 'Paluma' variety were offered to adults of *A. obliqua* every two days for oviposition and later removed and placed in plastic trays containing vermiculite to obtain larvae and puparia, which were placed in 500 mL plastic pots with a thin vermiculite layer and closed with paper towels, aiming at the emergence of adults.

Rearing of *D. longicaudata* was established from parasitized pupae of *C. capitata* from the Laboratory of Entomology of Embrapa Cassava and Fruits (Embrapa/CNPMF). Parasitoid rearing was maintained according to the methodology proposed by Carvalho et al. (1998), that is, third-instar larvae of *C. capitata* were used as hosts for the parasitoid, being placed in "parasitism units." These parasitism units consist of groups of 100 larvae of *C. capitata* wrapped in thin organza fabric fixed to the ceiling of 30 × 30 × 30 cm acrylic cages with the parasitoids. The units were periodically exposed to parasitoids around five days old for one hour. After the parasitism units were exposed, the larvae were placed in 500 mL plastic containers with vermiculite for pupation and emergence of adults. The adults were maintained in an acrylic cage (30 × 30 × 30 cm) with water and a diet based on distilled water, honey, agar-agar, ascorbic acid, and nipagin.

Toxic effects of botanical insecticides and extracts on adults of Anastrepha obliqua in a single dose

The experimental design was completely randomized, with 10 treatments and five replications, totaling 50 plots. Each plot consisted of a pot with 10 adult fruit flies. Treatments consisted of six extracts, two neem (*Azadirachta indica*)-based commercial products (Azamax® and Natuneem®), one ethanol treatment, and no application (control). The plant extracts consisted of a methanol extract of leaves of *Aspidosperma spruceanum* (MLAS), ethanol extract of bark of *Metrodorea maracasana* (EBMM), hexane extract of leaves of *Conchocarpus mastigophorus* (HLCM), ethanol extract of leaves of *Conchocarpus mastigophorus* (ELCM), ethyl acetate extract of leaves of *Xylopia sericea* (ALXS), and seed oil of *Aleurites moluccana* (OSAM). The extracts were coded using the initial letter of the solvent (ethanol, hexane, methanol, or ethyl acetate) as the first letter, the initial letter of the plant organ as the second letter, and the initials of the scientific name of the species as the third and fourth letters (Table 1).

Table 1
Botanical extract abbreviation, dry mass (g), extract mass (g), and yield (%) as a function of the plant species, botanical family, and plant organ used to obtain the extract.

Species	Family	Plant organ	Abbreviation	Dry mass (g)	Extract mass (g)	Yield (%)
Aspidosperma spruceanum	Apocynaceae	Leaf	MLAS	54.76	19.16	35
Aleurites moluccana	Euphorbiaceae	Seed	OSAM	198.03	99.67	50
Conchocarpus mastigophorus	Rutaceae	Leaf	ELCM	196.36	8.56	4
Conchocarpus mastigophorus	Rutaceae	Leaf	HLCM	196.36	3.92	2
Metrodorea maracasana	Rutaceae	Bark	EBMM	278.10	40.14	14
Xylopia sericea	Annonaceae	Leaf	ALXS	813.82	25	3

The plant extracts were obtained by the team from the Laboratory of Natural Products of UESB, campus of Jequié, BA, Brazil, and prepared from leaves and bark of the plant species. The plant material was dried in an oven at 40°C for 48 hours and subjected to cold extraction with hexane, ethyl acetate, or methanol by maceration, following the methodology described by Gomes (2014). The oil from seeds of *A. moluccana* was prepared as follows: the seeds were washed and dried in an oven at 50°C for 72 hours and then the seeds were cold-pressed for oil extraction using a hydraulic press with a capacity of 10 tons.

The extracts were evaluated for toxicity by topical application on fruit flies by adapting the methodology used by Siskos et al. (2008) and Efrom et al. (2011). The extracts were diluted using ethanol at a concentration of 70 mg mL^{-1}. The botanical insecticides were diluted according to the manufacturer's recommendation, that is, Azamax® at 5 mL L^{-1} and Natuneem® at 10 mL L^{-1}. Ten females of *A. obliqua* were used per plot, in which extracts, commercial products, and solvent were applied using a 10-µL graduated micro-syringe. The fruit flies were immobilized in a freezer for about two minutes before application and then 1.5 µL of extract solution was applied to their dorsal region. After application, the fruit flies were transferred to recipients with diet and a wad of cotton moistened with water. The evaluations were performed 24, 48, 72, and 96 hours after application. The total mortality and agronomic efficiency rates were calculated from the data obtained after the last evaluation (96 h), using the formula of Abbott (1925). Botanical insecticides or extracts that presented mortality higher than 50% were evaluated at different concentrations on adults of *A. obliqua*.

Effect of Metrodorea maracasana extract at different concentrations on adults of Anastrepha obliqua

The extract EBMM provided the highest percentage of mortality and, therefore, the only one used in the concentration study. The experimental design was completely randomized, with seven treatments and five replications, totaling 35 plots. The treatments consisted of concentrations of *M. maracasana* extract (60, 70, 80, 90, and 100 mg mL^{-1}), in addition to the solvent treatment (ethanol) and no application (control). The extracts were evaluated for toxicity by the topical application on fruit flies, using the same procedures and methodology described in the previous item.

Sublethal effects of botanical insecticides on adults of Anastrepha obliqua

The study of sublethal effects was carried out with commercial products based on neem and water, using the same procedures to test the toxic effect of botanical insecticides and extracts.

Fruit flies that survived insecticide applications after 96 hours were used for the bioassay. The experimental design was completely randomized, with seven treatments and four replications, totaling 28 plots. Grape berries of the cultivar Itália were used. The treatments consisted of two botanical insecticides, varying the application, namely: T1 – Natuneem® application on the fruit fly and fruit; T2 – Natuneem® application on the fruit; T3 – Natuneem® application on the fruit fly; T4 – Azamax® application on the fruit fly and fruit; T5 – Azamax® application on the fruit; T6 – Azamax® application on the fruit fly; T7 – water application (control). The botanical insecticides were diluted according to the manufacturer's recommendation: Azamax® at 5 mL L^{-1} and Natuneem® at 10 mL L^{-1}.

The grapes were immersed in the insecticide solution and placed to dry at room temperature for approximately 10 minutes. Three couples of *A. obliqua* were placed in plastic pots with water and an artificial diet based on sugar and yeast extract. Two grapes treated with insecticides were offered in each pot, remaining exposed for oviposition for 48 hours. After this period, the grapes were dissected to count and remove the eggs. A magnifying glass with a 20x magnification was used to visualize the eggs and a stylet and a brush were used to remove the eggs, which were placed in Petri dishes on filter paper stained with blue aniline for better visualization. The Petri dishes were placed in a climate-controlled room at a temperature of 23 ± 1°C and relative humidity of 70 ± 10%; the filter paper was moistened daily.

The Oviposition Preference Index (IPO) was calculated using the formula suggested by Fenomore (1980): $IPO = [(T - P) / (T + P)] \times 100$, where, T = number of eggs in the evaluated treatment and P = number of eggs in the control treatment. The index ranges from + 100 (very stimulating) to -100 (total deterrence/inhibition), with a value of 0 indicating no effect. Egg viability was estimated through daily evaluations for a period of five days, quantifying the number of hatched larvae.

Toxic effects of botanical insecticides and extracts on adults of Diachasmimorpha longicaudata

The experimental design was completely randomized, with six treatments and five replications, totaling 30 plots. Each plot consisted of 10 parasitoids (females). The treatments consisted of two extracts, that is, ethanol extract of leaves of *C. mastigophorus* (ELCM) and ethanol extract of bark of *M. maracasana* (EBMM), two neem-based commercial products, i.e., Azamax® (Azadiractin A/B 12g L^{-1}, DVA Brasil) and Natuneem® (emulsified oil from neem seeds, Natural Rural), an ethanol treatment, and no application (control). Neem-based products are widely used in toxicity studies on pests and natural enemies and, therefore, were used as standard treatments for comparison with extracts.

The plant material was collected and prepared as described for the bioassay of sublethal effects of botanical insecticides on adults of *A. obliqua*, following the methodology described by Gomes (2014).

The extracts were evaluated for toxicity by topical application on parasitoids by adapting the methodology used by Siskos et al. (2008). The extracts were diluted using ethanol at a concentration of 70 mg mL^{-1}, while the commercial botanical insecticides were diluted according to the manufacturer's recommendation for pest control, that is, Azamax® at 5 mL L^{-1} and Natuneem® at 10 mL L^{-1}. Ten females of *D. longicaudata* were used in each plot and the treatments were applied using a 10-µL graduated micro-syringe. The parasitoids were immobilized in a freezer for about one minute before application and then 1.5 µL of the extract solution was applied to their dorsal region. After application, the parasitoids were transferred to recipients with diet and a wad of cotton moistened with water. The evaluations were conducted at 24, 48, 72, and 96 hours after application. The data were analyzed considering the mortality rates corrected in relation to the control with ethanol, using the formula of Abbott (1925).

Sublethal effects of botanical insecticides on Diachasmimorpha longicaudata

The experimental design was completely randomized, with seven treatments and five replications, totaling 35 plots. The treatments consisted of two botanical insecticides (Azamax® and Natuneem®), varying the application, namely: T1 – Natuneem® application on the parasitism unit and parasitoid (double application); T2 – Natuneem® application on the parasitoid; T3 – Natuneem® application on the parasitism unit; T4 – Azamax® application on the parasitism unit and parasitoid (double application); T5 – Azamax® application on the parasitism unit; T6 – Azamax® application on the parasitoid; T7 – EBMM application on the parasitism unit and parasitoid (double application); T8 – EBMM application on the parasitoid; T9 – EBMM application on the parasitism unit; and T10 – water application (control). Only the extract EBMM was used in the sublethal effects test, as it has toxic action on two species of fruit flies of quarantine importance, showing promise for studies of substance isolation and formulation (Gomes 2014; Costa 2016).

The insecticides were applied on the parasitoid following the methodology of the previous experiment and, after four days, the surviving parasitoids were used in this experiment. Thirty third-instar larvae of *C. capitata* from the laboratory rearing were placed in organza fabric, forming the parasitism units, which were immersed in the products for 30 seconds. Subsequently, the units were dried at room temperature and placed in cages with five females and five males of the parasitoid aged ten days for one hour, adapting the methodology of França et al. (2010). After exposure, the parasitism units were transferred to plastic pots with vermiculite, closed with paper towels, and secured with rubber bands, with the daily observation of the emergence of parasitoids and/or fruit flies.

Statistical analyses

Data from the bioassay of toxic effects of botanical insecticides and extracts on adults of *A. obliqua* in a single dose were subjected to the likelihood ratio test for a generalized linear model (GLM) with a 5% significance level, using the R software version 3.2.2 (R Core Team 2015), seeking the statistical model that would describe the factors that influenced *A. obliqua* survival. The percentages of mortality of treatments in the bioassay on the effect of *M. maracasana* extract at different concentrations on *A. obliqua* were analyzed as a function of concentrations using regression analysis. The data were subjected to analysis of variance and Tukey's test at a 5% probability, using the ASSISTAT 7.7 Beta software.

An a priori orthogonal contrast was performed in the bioassay of sublethal effects of botanical insecticides on *A. obliqua* to verify differences between treatments, being evaluated six contrasts: 1 – (control T7 x treatments T1, T2, T3, T4, T5, T6); 2 – (double application T1 and T4 x single application T2, T3, T5, T6); 3 – (application on the fruit T2, T5 x application on the fruit fly T3, T6); 4 – (Natuneem® with double application T1 x Azamax® with double application T4); 5 – Natuneem® application on the fruit T2 x Azamax® application on the fruit T5); and 6 – (Natuneem® application on the fruit fly T3 x Azamax® application on the fruit fly T6).

Data from all evaluations were used to study the toxic effects of botanical insecticides on the parasitoid, seeking a statistical model that would describe the factors that influence the survival of *D. longicaudata*. A likelihood ratio test was also performed for a GLM (generalized linear model) with a 5% significance, using the R software version 3.2.2 (R Core Team 2015). The tested products were classified based on parasitoid mortality as: 1 – harmless (< 30% mortality); 2 – slightly harmful (30–79%); 3 – moderately harmful (80–99%); and 4 – harmful (> 99% mortality) (Sterk et al. 1999; Harbi et al. 2017).

Data on the sublethal effects of botanical insecticides on the parasitoid were analyzed considering the methodology proposed by Stupp et al. (2020) and Bernardi et al. (2019), which estimates the reduction in parasitism capacity for each treatment compared to the control, using the formula: $PR = [1 - (P / p) * 100]$, where PR is the percentage of parasitism reduction, P is the mean parasitism value for each product, and p is the mean parasitism observed for the control treatment. Each product was classified into the following classes depending on the results: 1 – harmless ($PR < 30\%$); 2 – slightly harmful ($30\% \leq PR \leq 79\%$); 3 – moderately harmful ($80\% \leq PR \leq 99\%$); and 4 – harmful ($PR > 99\%$) (Bernardi et al. 2019).

The data related to the number of emerged parasitoids were subjected to the Bartlett and Shapiro-Wilk tests to evaluate the assumptions of homoscedasticity of treatment variances and normality of residuals, respectively, and then subjected to analysis of variance to compare the means by Tukey's test ($P < 0.05$) using the R software version 3.2.2 (R Core Team 2015).

Results

Toxic effects of botanical insecticides and extracts on adults of Anastrepha obliqua in a single dose

In general, most of the studied products showed low toxicity to *A. obliqua*. All botanical insecticides and extracts differed from the control. The extract EBMM caused the highest percentage of mortality (74%) and the highest agronomic efficiency (73%) (Table 2), not differing from the percentages of mortality caused by Azamax® and the extracts ELCM and HLCM.

Table 2
Observed mortality (%) and total mortality (%) of *Anastrepha obliqua* and agronomic efficiency (%) of botanical extracts and insecticides at a concentration of 70 mg mL^{-1}.

Treatment*	Observed mortality (%)				Total mortality (%)**	Agronomic efficiency (%)
	24 h	48 h	72 h	96 h		
ALXS	4.0	6.0	8.0	12.0	12.0 b	8.3
OSAM	6.0	12.0	16.0	16.0	16.0 b	12.5
MLAS	2.0	4.0	16.0	16.0	16.0 b	12.5
EBMM	56.0	66.0	72.0	74.0	74.0 a	73.0
ELCM	36.0	38.0	38.0	38.0	38.0 ab	35.4
HLCM	22.0	22.0	24.0	26.0	26.0ab	23.0
Azamax®	12.0	16.0	24.0	28.0	28.0ab	25.0
Natuneem®	6.0	10.0	12.0	16.0	16.0 b	12.5
Control ethanol	2.0	2.0	4.0	6.0	6.0bc	–
Control	2.0	2.0	2.0	4.0	4.0c	–

*ALXS – ethyl acetate extract of leaves of *Xylopia. sericea*; OSAM – oil of seeds of *Aleurites moluccana*; MLAS – methanol extract of leaves of *Aspidosperma spruceanum*; EBMM – ethanol extract of bark of *Metrodorea maracasana*; ELCM – ethanol extract of leaves of *Conchocarpus mastigophorus*; HLCM – hexane extract of leaves of *C. mastigophorus*; Control – no application
**Means followed by the same letter do not differ from each other by Tukey's test at 5% probability.

The extracts of *X. sericea*, *A. moluccana*, *A. spruceanum*, and Natuneem® caused lower mortality in females of *A. obliqua* and showed mortality statically similar to the ethanol solvent.

The survival of *A. obliqua* varied among extracts, and the model with the best fit found in the likelihood test was Survival ~ Time + Azamax® + EBMM + ELCM + HLCM, in which survival occurs as a function of time interval and between extracts that showed significant results (Table 3).

Table 3
Observed mortality (%) and total mortality (%) of *Anastrepha obliqua* and agronomic efficiency of the ethanol extract of bark of *Metrodorea maracasana* (EBMM) as a function of concentrations.

Treatment	**Observed mortality (%)**				**Total mortality (%)***	**Agronomic efficiency (%)*****
	24 h	**48 h**	**72 h**	**96 h**		
EBMM 60 mg	32	54	54	58	58 b	56.2
EBMM 70 mg	38	44	60	70	70 a	68.7
EBMM 80 mg	46	62	68	72	72 a	70.8
EBMM 90 mg	56	70	72	80	80 a	79.2
EBMM 100 mg	60	74	78	80	80 a	79.2
Control ethanol	2.0	6.0	8.0	10	10 c	-
Control*	0.0	2.0	2.0	2.0	2.0 c	-
CV (%) =					27.62	

*Control treatment – no application.

**Means followed by the same letter do not differ from each other by Tukey's test at a 5% probability.

***Agronomic efficiency obtained by the Abbott formula (%).

Effect of Metrodorea maracasana extract at different concentrations on adults of A. obliqua

All concentrations differed significantly from the control treatment, and only the treatment with 60 mg mL^{-1} differed from the other concentrations. All concentrations showed mortalities higher than 50% (Table 4).

Table 4
Oviposition preference index (OPI) and classification of treatments as a function of the products Azamax® and Natuneem®.

Treatment	Oviposition preference index	Classification
T1 – Natuneem® application on fruit and fruit fly	−82.6	Deterrent
T2 – Natuneem® application on fruit	−71.4	Deterrent
T3 – Natuneem® application on fruit fly	−84.6	Deterrent
T4 – Azamax® application on fruit and fruit fly	−97.6	Deterrent
T5 – Azamax® application fruit	−82.6	Deterrent
T6 – Azamax® application on fruit fly	−82.6	Deterrent
T7 – Control (water)	0	Neutral

The concentrations 70, 80, 90, and 100 mg mL^{-1} showed agronomic efficiency equal to or higher than 70%, not significantly differing from each other.

Regression analysis indicated that increasing concentration leads to increases in the percentage of mortality up to a concentration of 90 mg mL^{-1} (Fig. 1).

Sublethal effects of botanical insecticides on adults of Anastrepha obliqua

A difference was observed in the number of eggs as a function of treatments ($F = 3.577$; $DF = 6.21$; $P = 0.016$). Only contrast 1 (control – T7 x treatments – T1, T2, T3, T4, T5, T6) was significant for both the number of eggs and egg viability, indicating that all treatments with botanical insecticides reduced oviposition (Fig. 3). The control treatment resulted in a higher number of eggs, with a mean of 21 eggs during the evaluation period. Azamax® (T4) applied on the fruit fly and fruit resulted in a mean laying of 0.25 eggs.

Egg viability was higher in the control treatment, with a mean of 77%, differing significantly from the other treatments ($F = 23.32$; $DF = 6.21$; $P = < 0.001$), which had the viability lower 10%. The oviposition preference index (OPI) by *A. obliqua* in grapes showed an oviposition inhibition effect in all treatments (Table 4).

Toxic effects of botanical insecticides and extracts on adults of Diachasmimorpha longicaudata

The statistical model found in the likelihood test with the best fit of the data was Survival ~ Time + Azamax® + Natuneem® + EBMM, in which survival occurs as a function of time interval and between the extracts that presented significant results (Table 5).

Table 5
Likelihood ratio test (LRT) for the logistic model of *Diachasmimorpha longicaudata* survival as a function of botanical insecticides and extracts.

Source of variation	G.L.	LRT	P
Azamax®	1	22.768	< 0.0001***
Natuneem®	1	9.2009	0.002419**
EBMM	1	6.6179	0.0101*
ELCM	1	0.23474	0.628
Control (ethanol)	1	1.4325	0.2314
Time	4	80.189	< 0.0001***

Significant at 0.1% (***), 1% (**) and 5% (*).

The selection of this model indicates that Azamax®, Natuneem®, and EBMM were toxic to the parasitoid *D. longicaudata*, providing a lower survival compared to the control, with values of 58, 76, and 80%, respectively, over the parasitoid adults (Fig. 3). The extract ELCM provided the highest percentage of survival (92%), similar to the control treatment. Azamax® was classified as slightly harmful (class 2) and Natuneem®, EBMM, and ELCM were classified as harmless (class 1) based on the percentages of parasitoid mortality (Table 6). The highest mortality rates were recorded within 24 hours for all botanical extracts and insecticides, mainly for Azamax®, reaching 28% mortality (Table 6).

Table 6

Observed mortality (%) as a function of evaluation time (hours), total mortality (%), and corrected mortality (%) of *Diachasmimorpha longicaudata* and classification of botanical insecticides and extracts.

¹Treatment	Observed mortality (%)				Total mortality (%)	²Corrected mortality (%)	³Classification
	24h	48h	72h	96h			
Azamax®	28.0	34.0	34.0	42.0	42.0	40.8	2
Natuneem®	22.0	22.0	24.0	24.0	24.0	22.4	1
EBMM	18.0	18.0	20.0	20.0	20.0	18.4	1
ELCM	2.0	6.0	8.0	8.0	8.0	6.1	1
Control (ethanol)	0.0	2.0	4.0	6.0	6.0	-	-
Control	2.0	2.0	4.0	4.0	4.0	-	-

[1]Treatment: EBMM – ethanol extract of bark of *Metrodorea maracasana*, ELCM – ethanolic extract of leaves of *Conchocarpus mastigophorus*.

[2]Corrected mortality obtained by the Abbott formula (%).

[3]Classification: 1 – harmless (< 30% mortality); 2 – slightly harmful (30–79%); 3 – moderately harmful (80–99%), and 4 – harmful (> 99% mortality).

Sublethal effects of botanical insecticides on Diachasmimorpha longicaudata

The number of emerged parasitoids was influenced by the interaction control × factor ($F = 18.4479$; $P < 0.0001$), with means of 10.20 and 6.35, respectively (Table 7). The slicing of the interaction for the products applied only on the parasitoid showed that EBMM differed from the other products ($F = 28.638$; $P < 0.0001$), presenting a higher emergence of parasitoids, with a mean of 23.0, followed by Azamax® with 6.2, which did not differ from Natuneem® with 5.8 parasitoids. Products applied on parasitism units showed that the extract EBMM provided the highest emergence of the parasitoid (7.4), not differing from Azamax® (4.4) and Natuneem®, which presented the lowest number of parasitoids (0.6) ($F = 3.3124$; $P = 0.0467$). Considering only the double application, treatments based on neem and the extract EBMM had a similar effect relative to the number of parasitoids ($F = 1.6885$; $P = 0.1977$) (Table 7).

Table 7
Mean number of emerged parasitoids, reduction in parasitism capacity (PR), and classification.

Treatments	Parasitoid (N°)	PR (%)	Classification*
T1 – Natuneem® double application	1.6 ± 2.3	84.3	3
T2 – Natuneem® application on parasitoid	5.8 ± 3.0	43.1	2
T3 – Natuneem® application on parasitism unit	0.6 ± 1.3	94.1	3
T4 – Azamax® double application	6.4 ± 3.9	37.2	2
T5 – Azamax® application on parasitoid	6.2 ± 3.4	39.2	2
T6 – Azamax® application on parasitism unit	4.4 ± 6.3	56.9	2
T7 – EBMM double application	1.8 ± 3.49	82.3	3
T8 – EBMM application on parasitoid	23.0 ± 1.41	1.25	1
T9 – EBMM application on parasitism unit	7.4 ± 1.01	27.4	1
T10 – Control	10.2 ± 3.5	–	–

[3]Classification: 1 – harmless (PR < 30% mortality); 2 – slightly harmful (30% < PR < 79%); 3 – moderately harmful (80% < PR < 99%), and 4 – harmful (PR > 99% mortality).

The analysis of the type of application for each product showed that only EBMM application was significant ($F = 29.0818$; $P < 0.0001$), that is, this extract provided higher emergence of parasitoids when the application was only on the parasitoid, with a mean of 23.0, differing from the application on the parasitism unit (7.4) and the double application (1.8).

The number of fruit flies presented differences between the control and factorial ($F = 10.3236$; $P < 0.0001$), with a higher mean of the emergence of fruit flies in the control treatment (13.0) relative to the factorial (6.35) (Table 7). The slicing of the interaction product within application level showed that the extract EBMM and Natuneem® did not differ from each other in the double application, with mean emergences of 27.0 and 23.6 fruit flies, respectively, and both products differed from Azamax® (2.6 fruit flies) ($F = 26.5481$; $P < 0.0001$). The products presented no effect on the number of fruit flies emerged when considering application only on both parasitoid ($F = 1.5653$; $P = 0.2216$) and parasitism unit ($F = 3.0707$; $P = 0.575$).

The application effect on each product showed that the double application of EBMM ($F = 26.9595$; $P < 0.0001$) provided the highest emergence of fruit flies, with a mean of 27.0, differing from the application in the parasitism unit, with a mean of 16.8, and the application on the parasitoid, with a mean of 0.2 (Table 7). The type of application of the products Azamax® and Natuneem® did not affect the emergence of fruit flies.

Treatments (T4 and T6) with Azamax® provided a high rate of unviable puparia (around 70%) compared to the other treatments. The treatments Natuneem® T1 – double application and T3 – application on the parasitism unit stood out among the insecticides that affected the parasitism of *D. longicaudata* on larvae of *C. capitata*, which reduced parasitism by 84.3 and 94.1%, respectively (Table 7). The extract EBMM reduced parasitism only in the double application (T7) by approximately 82.3%. Based on the reduction of parasitism capacity, the extract EBMM applied on the parasitoid (T8) and parasitism unit (T9) was considered harmless (class 1), all treatments with Azamax® (T4, T5, and T6) and Natuneem® applied on the parasitoid (T2) were classified as slightly harmful (Class 2), and Natuneem® with the double application and application on the parasitism unit (T1 and T3) and EBMM also with the double application were moderately harmful (Class 3).

Discussion

In general, only the extract of *M. maracasana* (EBMM) caused the highest mortality in adults of *A. obliqua*. Similarly, Gomes (2014) used the same extract at a concentration of 50 mg mL^{-1} and found a 76% mortality for *C. capitata*.

Extracts obtained from leaves of *C. mastigophorus* (ELCM and HLCM), in which the type of solvent for extraction varied, showed no difference between the percentages of mortality in *A. obliqua*. The toxic effect on *A. obliqua* may be related to the main secondary metabolites found in this plant species, such as alkaloids, coumarins, and lignans. The solvent used in the extraction is another factor to consider. According to Torres et al. (2001), the susceptibility of insect pests to allelochemicals extracted from plants depends on the organ, plant species, mode of extraction, and target insect. However, the solvent used in the present study did not significantly affect the susceptibility of fruit flies to the extracts.

The low toxicity of *X. sericea* (ALXS) on *A. obliqua* may be related to the quantity of volatile components in the leaf. According to Pontes et al. (2007), monoterpenes and sesquiterpenes present in the oil of fruits and leaves have proven their action on the mite *Tetranychus urticae* Koch.

Regarding the commercial products, Azamax® presented significant mortality compared to the control, but it was low in comparison with EBMM. On the other hand, Natuneem® did not show significant mortality, in agreement with Efrom et al. (2011) for Natuneem® and Organic Neem®, which did not result in a significant mortality effect on adults of *A. fraterculus*, and Azevedo et al. (2013), also using the product Natuneem® on *Anastrepha* spp. In contrast, Machota Junior et al. (2013) observed that the product Rot-Nim® caused 86% mortality in adults of *A. fraterculus* at two hours after application.

All concentrations of the extract EBMM in the bioassay showed agronomic efficiency (corrected mortality) equal to or higher than 70%, not significantly differing from each other. Similarly, Gomes (2014) studied several plant extracts and found that the extract of bark of *M. maracasana* was the most effective on *C. capitata*. According to the author, pyranocoumarin substances are probably responsible for the toxicity of *M. maracasana* to *C. capitata*.

Another fact is related to an increase in concentration, leading to increases in the percentage of mortality up to the concentration of 90 mg mL^{-1} (Fig. 1). Concentrations equal to or higher than 90 mg mL^{-1} led to mortality stabilization and, therefore, the use of concentrations higher than this is not necessary.

Field studies should be carried out to confirm the efficacy of extract of *M. maracasana* at a concentration of 70 mg mL^{-1} on *A. obliqua*, as it has statistically equal mortality at higher concentrations.

All neem-based treatments reduced oviposition and egg viability compared to the control. According to Silva et al. (2012), azadirachtin has a repellent action on oviposition. The results obtained in the present study indicated that neem-based products, when deposited both on the fruit fly and the fruit, have the potential to reduce the oviposition of fruit flies, protecting the orchard, which is confirmed by the low oviposition preference index (OPI). The lower number of eggs deposited on the fruits was due to the inhibition of oviposition. The plant is rejected by the pest when oviposition deterrence occurs, which leads only to puncture in the fruit or a lower number of eggs per laying (Silva 2010).

Deterrence was not total, but resulted in high oviposition inhibition rates, being an important tool for fruit fly management when using azadirachtin-based products (Mahmoud and Shoeib 2008; Silva et al. 2012).

Although most treatments have led to low mortality of adults of *A. obliqua*, knowledge about the likely sublethal effects of botanical insecticides and extracts is essential for the selection of compounds for use in integrated pest management programs.

The toxicity obtained for adults of the parasitoid *D. longicaudata* allowed classifying the extracts EBMM and ELCM and Natuneem® as harmless and Azamax® as slightly harmful. These results are of paramount importance for the biorational management of insect pests because studies with fruit flies have shown toxic effects of these extracts on pests of economic and quarantine importance worldwide, such as *C. capitata* (EBMM) (Gomes 2014) and *A. obliqua* (EBMM and ELCM) (Costa 2016), especially EBMM, responsible for corrected mortality rates of fruit flies of 77.7 and 73.0%, respectively. This selectivity has already been observed for the same parasitoid when exposed to extracts of seeds of *Annona mucosa* Jacq (Stupp et al. 2020).

The effects of botanical extracts or insecticides on parasitoids vary as a function of the type of extract, active substance concentration, targets and their stages of development, and exposure methodology, among others. Thus, selectivity in favor of parasitoids to neem-based products may (Viñuela et al. 2001; Silva and Bueno 2015; Silva et al. 2016; Asma et al. 2018) or may not be observed (Gonçalves-Gervásio and Vendramim 2004; Nogueira et al. 2011; Harbi et al. 2017).

Regarding the evaluation time, the results showed that mortality was significantly higher in the first evaluation (24 hours after topical application) than the others, allowing the discrimination between treatments with only one evaluation. This result was similar to that obtained by other researchers for *D.*

longicaudata (Bernardi et al. 2019) and may help in the screening phase of bioactive compounds, leading to time and work effort optimization.

The cleaning behavior, that is, the act of trying to remove liquids from the body was an interesting aspect observed right after the topical application of treatments on the parasitoid tergum. This behavior was observed by Ruiz et al. (2008) in studies with topical application of the organosynthetic insecticide spinosad on *D. longicaudata*, which led the authors to raise the hypothesis that this behavior may lead to a reduction in deposition of the product on the insect's body, reducing its lethal effect. Self-cleaning is a common behavior in social insects, mainly ants, allowing them to remove potentially pathogenic organisms or substances present on their body surface (Camargo et al. 2017). Some parasitoids of the family Braconidae may also exhibit this behavior (Bilodeau et al. 2013). The ideal when selecting an extract, compound, or substance with bioactivity against insect pests would be the association between high toxicity and high selectivity to natural enemies and the indication as a potential formulation those that associate these two characteristics. Considering global trends in the management of agricultural and forest pests, biorational products must be aligned with the trend of compatibility with natural biological control and applied to the detriment of organosynthetic products of high toxicity, broad spectrum of action, and persistence in the environment. However, most of the studies are restricted to the toxic action on the pest and natural enemy, while the sublethal effects on natural enemies have secondary importance in the generation of knowledge

The sublethal effects caused by botanical insecticides and extracts are highly variable among non-target species, standing out the reduced longevity, fecundity, pupal viability, parasitism capacity, and malformations (Alvarenga et al. 2012; Fontes et al. 2018; Parreira et al. 2018). In the present study, the reduction in parasitism capacity was quantified as a function of the parasitoid phase exposed to the treatment, that is, adult and larva (parasitism unit). No treatment caused a reduction in the parasitism capacity considering the application only on adults, while Natuneem® drastically reduced the parasitism capacity (94.1%) when the application was carried out only on the parasitism unit (larvae), and the other treatments did not cause this effect. Natuneem® and the extract EBMM caused a reduction in parasitism capacity of 84.3 and 82.3%, respectively, under the double application condition, i.e., the exposure of both phases (adult and larvae) to the treatments. This condition simulated, for example, a full coverage application of the botanical extract and insecticide in the field, reaching parasitoid adults and fruits and, indirectly, probable parasitoid larvae inside fruit fly larvae that feed on the fruit pulp. In this case, the treatments must have a translaminar effect to reach the probable parasitoid larvae. However, the translaminar effect of this extract has not been proven (EBMM). In contrast, Azamax® was harmless in terms of sublethal effects under all application conditions. This difference in the reduction of parasitism capacity among neem-based botanical insecticides may be related to the composition, formulation, and concentration of the active ingredient of products (Hohmann et al. 2010). Natuneem® may have caused repellency, as it has azadirachtin and other bioactive substances that exude a strong odor when diluted, according to the manufacturer (Natural Rural), which may interfere with parasitism since the parasitoid uses visual and olfactory stimuli at short distances for host selection (Vinson 1976).

On the other hand, Smaniotto et al. (2013) observed that Natuneem® did not affect the percentage of parasitism and emergence of adults of *Telenomus podisi* Ashmead.

The mode in which the product is applied may also interfere with the reduction in parasitism (Viñuela et al. 2001). The parasitism capacity of females of *Opius concolor* (Szèpligeti) was reduced by the residual contact and ingestion of azadirachtin by 40.5 and 32.5%, respectively, whereas by the topical contact showed no interference in this parameter (Viñuela et al. 2001). The effects of neem applied only on hosts (eggs or larvae) are the ones that most affect parasitism in *Trichogramma achaeae* Nagaraja and Nagarkatti (Fontes et al. 2018), *Trichogramma galloi* Zucchi (Parreira et al. 2018), and *D. longicaudata* (France et al. 2010). The exposure of already parasitized fruit fly larvae to neem also affects the emergence of adult parasitoids (Alvarenga et al. 2012).

The greatest advantages among the studied treatments fall on the extract EBMM, as it is harmless to adults of *D. longicaudata* and does not determine a sublethal effect when applied only on adults or parasitism units (larvae), whereas neem-based products have been toxic for adults (Azamax®) or caused a significant reduction in parasitism (Natuneem®). The double application (adult and parasitism unit) of EBMM and Natuneem® reduced the parasitism capacity of *D. longicaudata*, being considered moderately harmful, which is a maximum exposure condition. In addition to the toxic or sublethal effects caused by neem products on *D. longicaudata*, these insecticides had low agronomic efficacy (12.5% for Natuneem® and 25.5% for Azamax®) to control *A. obliqua* (Costa 2016), adding disadvantage to these products.

Chemical studies have indicated that *M. maracasana* is a plant species rich in coumarins, mainly pyranocoumarins (Reis et al. 2010), probably responsible for bioactivity. Isolated fractions of this substance, such as isodentatin and citrusarin A and the mixture containing citrusarin A + dipetalolactone + isodentatin, provided mortalities of 100, 96.7, and 96.7% in *C. capitata*, respectively (Gomes 2014).

In summary, the ethanol extract of bark of *M. maracasana* (EBMM), ethanol extract of leaves of *C. mastigophorus* (ELCM), and hexane extract of leaves of *C. mastigophorus* (HLCM) are toxic to adults of *A. obliqua*. The ethanol extract of bark of *M. maracasana* (EBMM) has the potential to control *A. obliqua*, being promising for substance isolation and formulation studies. The insecticides Azamax® and Natuneem®, sprayed on grape berries, caused inhibition of oviposition and reduced egg viability of *A. obliqua*. The extract EBMM has the potential for bioprospecting, with fractionation and detection of bioactive substances for the main species of fruit flies for the later formulation, aiming at the development of a botanical insecticide with high toxicity to fruit flies and selectivity to *D. longicaudata*, well as studying the its probable effect translaminar effect.

Declarations

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Conflicts of interest

The authors declare that they have no known competing financial intersts or personal relationships that could have appeared to influence the work reported in this paper.

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Figures

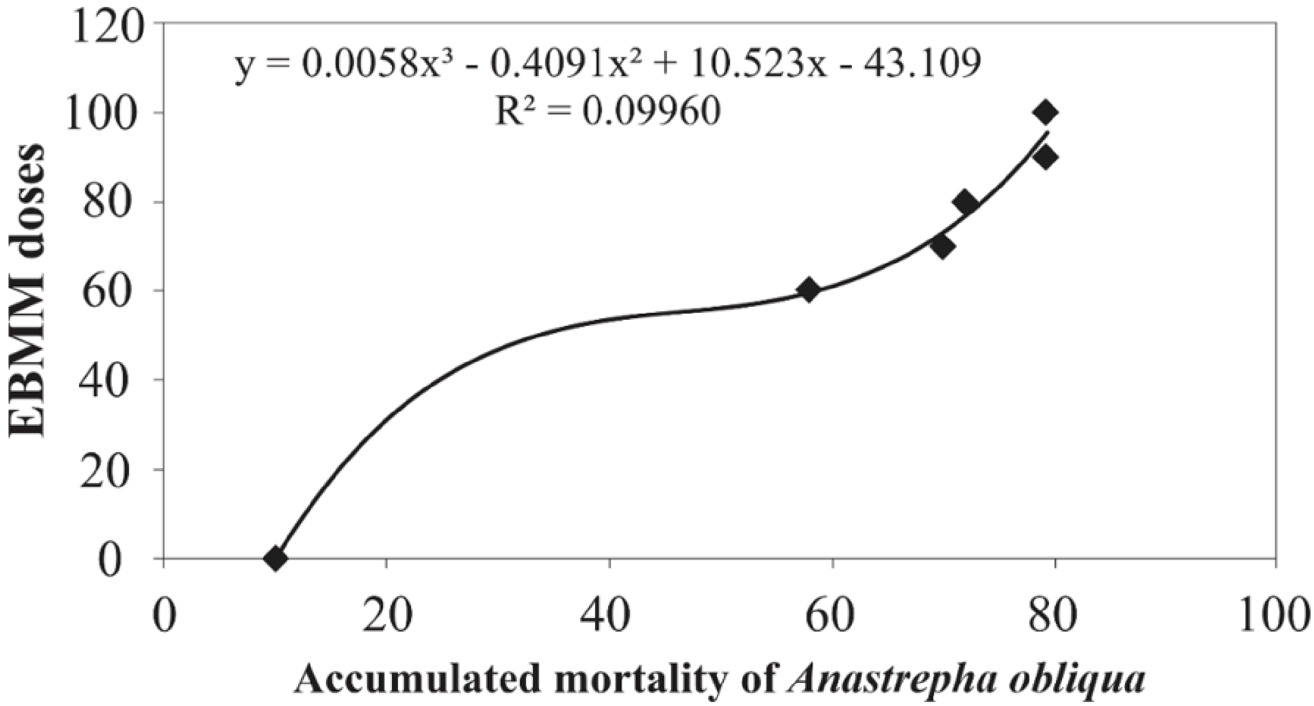

Figure 1

Estimated mortality of adults of *Anastrepha obliqua* as a function of concentrations (0, 60, 70, 80, 90, and 100 mg mL^{-1}) of the extract of bark of *Metrodorea maracasana*.

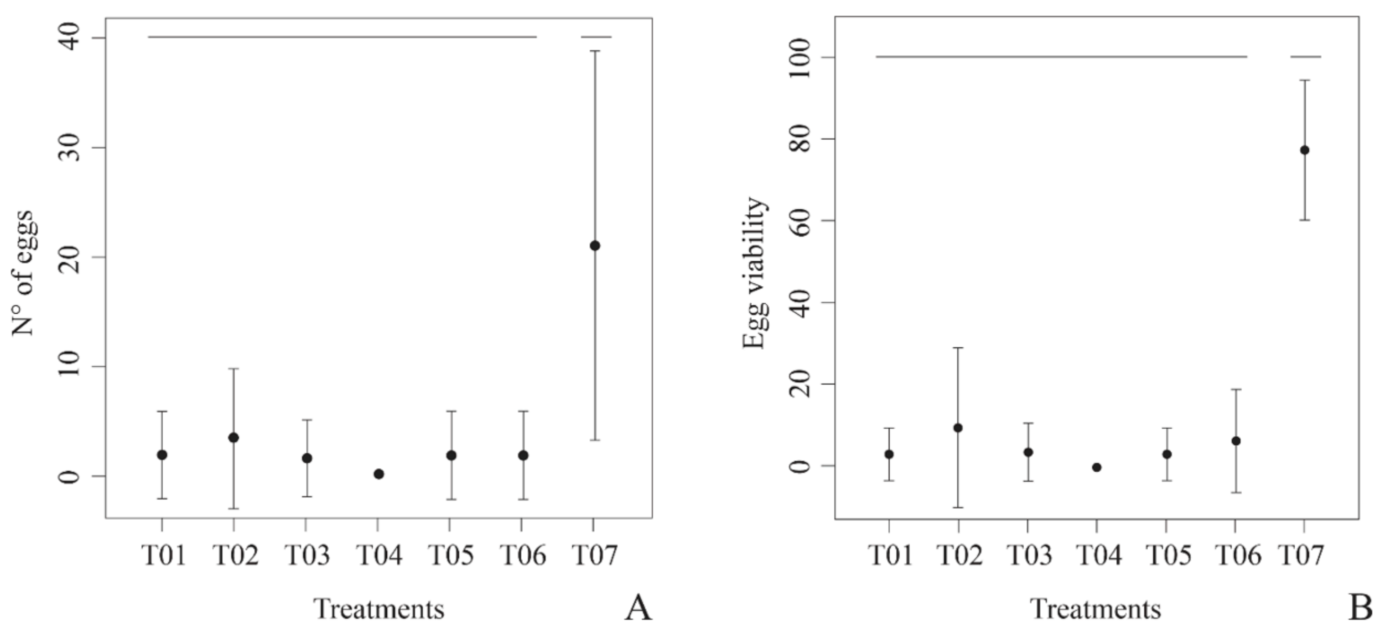

Figure 2

Mean number (A) and viability of eggs (B) of *Anastrepha obliqua* after application of botanical insecticides (Azamax® and Natuneem®) on grape berries and adults of *Anastrepha obliqua*. Treatments: T1 – Natuneem® application on the fruit fly and fruit, T2 – Natuneem® application on the fruit, T3 – Natuneem® application on the fruit fly, T4 – Azamax® application on the fruit fly and fruit, T5 – Azamax® application on the fruit, T6 – Azamax® application on the fruit fly, and T7 – control (water). $F=23.32$; $DF=6, 21$; $P<0.001$.

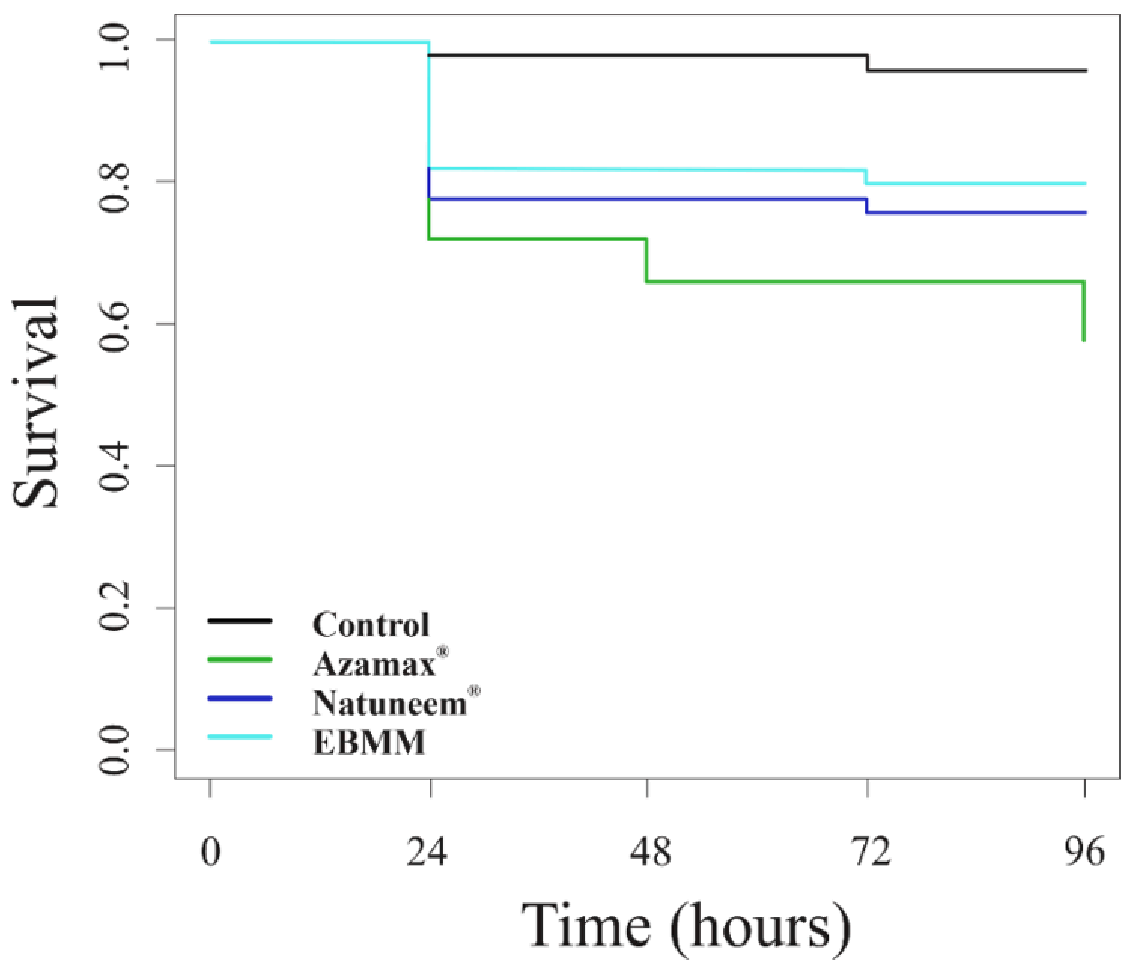

Figure 3

Estimated survival of *Diachasmimorpha longicaudata* as a function of botanical insecticides and extracts.