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Effect Toxic and Behavioral of *Annona mucosa* (Annonaceae) on the Tomato Leaf Miner

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Abstract

Tuta absoluta (Meyrick) is considered a pest with high destructive potencial and its control depends mainly on successive applications of insecticides. Therefore, new alternatives for the control of the tomato leaf miner using plants with insecticidal potential have been examined. This study was aimed at evaluating the toxic effect of *Annona mucosa* extract on the developmental stages of *T. absoluta*. Larval survival bioassay was performed in which newly-hatched caterpillars were inoculated in tomato leaflets sprayed with *A. mucosa* extract in the LC_{50} and LC_{90} treatments and the insecticidal controls chlorfenapyr, methanol, and water. To identify the mode of action of the extract in caterpillars, histological analyzes of the integument and gut were carried out. To evaluate ovicidal activity and oviposition repellency, only the LC_{50} treatment and controls (water and methanol) were carried out. In the ovicidal bioassay 75 eggs/treatment were used, and for the oviposition repellency, 10 couples/treatment, with 10 replicates. In the larval survival bioassay, a significant difference among survival curves, and the crude extract of *A. mucosa* significantly reduced the survival of *T. absoluta* caterpillars. The mode of action of the extract occurred by contact and ingestion, as indicated by changes in the integument and gut. The extract of *A. mucosa* also interfered in the embryonic development of *T. absoluta*, with a viability of more than 90% of the eggs. Regarding the behavioral effect, the extract reduced oviposition rates of *T. absoluta* females. Thus, *A. mucosa* extract had toxic effects on the different stages of pest development.

Keywords: *Tuta absoluta*, lethal concentration, toxicity, physiology, prospecting

1. Introduction

The tomato leaf miner, *Tuta absoluta* (Meyrick, 1917) (Lepidoptera: Gelechiidae) is an economically significant pest of tomato (Desneux et al., 2010), as it causes damage at all stages of plant development.

The control of this pest insects is carried out through successive applications of synthetic insecticides. However, this method has been inefficient, due to the high reproductive potential, short generation time and the leaf miner behavior and formation of galleries in fruits (Ortega, 2013; Lietti, Botto, & Alzogaray, 2005; Silva et al., 2011; Roditakis et al., 2015), which can generate losses of up to 100% of the production in tomato crops when there is no effective control (Lopez, 1991). The excessive use of insecticides also has adverse effects on the natural control of pests insect, environmental pollution and damage to human health (Picanço, Bacci, Crespo, Miranda, & Martins, 2007; Aktar, Sengupta, & Chowdhury, 2009).

In contrast to the problems presented, there is a need to search for safer forms to be used in the control of this pest, through the use of bioactive substances in plants which chemical characteristics potential for the synthesis of new products.

In this context, insecticidal plants can be an alternative for playing an important role in the sustainable control of insect pests, since they have combinations of complex molecules that allow biological effects to be the result of a

synergism between all of them, unlike synthetic insecticides in which one or a few molecules cause the observed effects (Pino, Sánchez, & Rojas, 2013; Zoubiri & Baaliouamer, 2011).

The effects of the secondary metabolites derived from insecticidal plants have several biological activities, such as larvicidal action, ovicides, interference of oviposition behaviour, repellency, among others (Gokçe, Issacs, & Whalon, 2011; Chean, Tay, & Chan, 2013; Jeyasankar, Elumalai, Raja, & Ignacimuthu, 2013). This effects can be observed with histological analysis that allow to verify and to describe the changes cell in differents tissues ofthe insects (Correia, V. W. Texeira, A. A. C. Texeira, Oliveira, & Torres, 2009).

Among the species of insecticidal plants that show biological activities in the control of insect pests, *Annona mucosa* Jacq. (Annonaceae), stands out for showing promising results with different modes of action, such as, ingestion action on caterpillars *Chrysodeixis includens* Walker (Lepidoptera: Noctuidae) (Massarolli, Pereira, & Foerster, 2016) and *Spodoptera frugiperda* Smith (Lepidoptera: Noctuidae) (Ribeiro, Ansante, & Vendramim, 2016) and ovicide and behavioural activities on *Plutella xylostella* L.(Lepidoptera: Plutellidae) (Souza et al., 2015).

In view of the results that confirm the insecticidal action of *A. mucosa*, the aim of this study was to test the toxic effect of this species and the possible changes cells in caterpillars in laboratory bioassays.

2. Material and Methods

2.1 Rearing of Tomato Leaf Miner

The rearing was started with caterpillars and pupae collected in commercial tomato plantations in the municipality of Tangara da Serra, Mato Grosso State. After collection, the insects were kept in an air-conditioned room in the laboratory, under controlled conditions (Temperature of 25±1 °C, Relative Humidity of 60±10%, and fotofase of 12 hours). The method of rearing was an adaptation of the methodology proposed by Krechemer (2010), in which pet bottles were used with the bottoms cut, and soon inserted tomato leaves, to Santa Cruz cultivars, to feed the caterpillars. This pet bottles which were covered with voil to avoid their escape. The leaf base was dipped in a plastic tube, which contained water to maintain the turgescence, and every two days healthy tomato leaves were offered for feeding caterpillars.

The pupae were removed and destined for the wooden cage coated with voil. After the emergence of the adults tomato leaflets were inserted into the oviposition, with the petiole dipped in a vial with water to maintain the turgescence of the leaf. Every two days the leaves were replaced, and the eggs transferred to transparent plastic pots until hatching.

2.2 Obtaining the Extract of A. mucosa

The fruits of *A. mucosa* were collected in the periurban area of Tangara da Serra-MT (latitude of 14°37′55″S and longitude 57°28′05″W) and deposited in the Herbarium of the State University of Mato Grosso in Tangara da Serra (Voucher 964). In the entomology laboratory, the fruits were pulped and the seeds dried in an oven with regulated air circulation (40 °C) for 72 hours. Afterwards the seeds were ground in a knife mill to obtain a fine powder. This powder was mixed with methanol 1:3 (extract:solvent) and percolated for 72 hours. After this period the mixture was filtered and the solvent evaporated in a rotary evaporator at a temperature of 40 °C under reduced pressure (Piton, Turchen, Butnariu, & Pereira, 2014). With the remaining residue the process was repeated three times, and at the end the extract was collected in a single container totaling 201,45 g. The extract obtained showed dark coloration and aspects of density and oiliness characterized by its apolarity (lipophilicity). From the crude extract the concentrations used in the conduction of the bioassays were obtained, in which methanol was used as the solvent.

The application of the treatments was done by Arprex spray gun, model 5AD, gravity type, coupled to a direct air compressor. The sprays were made to the point of draining and the leaflets and/or plants remained at room temperature for 15 minutes. After application of the treatments, the petiole of the leaflets were wrapped with humidity cotton to maintain the turgescence.

2.3 Concentration-Mortality Bioassay

To determine the lethal concentrations (LC 50 and 90) of the *A. mucosa* extract on *T. absoluta* caterpillars, the concentration-mortality test was performed. Newly hatched larvae were submitted to different concentrations of the extract (*i.e.* 100, 50, 25, 15, 10 and 5 ppm) and to the control (methanol). In this procedure, the leaflets of the middle third of the tomato leaves were sprayed with the concentrations and, subsequently, a newly hatched caterpillar was inoculated by leaflet (n = 20 larvae/treatment). Next were then transferred to 150 ml plastic pots

where they remained for 24 hours. After this period the mortality was recorded and the data were used to estimate the lethal concentrations, being used as reference for the other bioassays.

2.4 Acute Toxicity of A. mucosa on T. absoluta Caterpillars

In this bioassay was used the lethal concentration (LC50 and LC90) of the *A. mucosa* extract, the insecticide chlorfenapyr as a positive control and methanol and water were used as negative controls. An adaptation of the methodology of Ferreira, Vendrami and Forin (2012) was used, spraying 1.5 ml of each treatment per leaflet, for a total of 20 replicates. After drying the products, a caterpillars was inoculated with leaflets and packed in transparent plastic bottles of 150 ml. The leaflets were replaced every three days with a new untreated. Evaluations were performed daily until mortality or pupation.

2.5 Histological Analysis of the Action of A. mucosa on T. absoluta Caterpillars

To verify the mode of action of the extract, tomato leaflets were sprayed with the LC50, then first instar caterpillars were added, which remained in contact with the leaf for 24 hours after application. The same procedure was performed with the water control.

The histological analyzes followed the protocols of the Laboratory of Insect Tissue Biology of the State University of Londrina (UEL), with whole caterpillars fixed in Karnovsky solution (2.5% glutaraldehyde + 4.0% paraformaldehyde in 0.1 M phosphate buffer and pH 7.2) for 6 hours. After this time the material was washed in 0.1 M sodium phosphate buffer and pH 7.2 (5 minutes), followed by dehydration in 70% ethyl alcohol (5 minutes), ethyl alcohol 90% (20 minutes) and ethyl alcohol 100% (20 minutes). After this procedure, the material was pre-infiltrated in resin + ethyl alcohol solution (1:1) for 4 hours at room temperature, basic resin infiltration + activator at room temperature for 24 hours and inclusion on appropriate polyethylene molds. After the polymerization was complete, the blocks were cut in microtome and the sections (5 μm) were fixed on slides, stained with Hematoxylin and Eosin (HE) and photographed.

2.6 Toxicity of A. mucosa on T. absoluta Eggs

To evaluate the ovicidal effect of the *A. mucosa* extract, tomato leaves were inserted into the adult cage for 24 hours, according to the methodology of Trindade, Marques, Xavier, and Oliveira (2000). After this period, 20 leaflets containing 15 eggs each (repetitions) were separated, totaling 75 eggs/treatment.

On the leaflets were sprayed 1.5 ml of solution corresponding to 21.13 ppm of the *A. mucosa* extract, and chlorfenapyr, methanol and water controls, totaling four treatments with five replicates each. Evaluations were performed daily, recording of embryonic development and/or egg inviability.

2.7 Behavioral Bioassay: Oviposition Preference

The preference of *T. absoluta* adults for oviposition was performed in bioassays with a chance of choice, adapting to the methodology of Ribeiro et al. (2015).

In this bioassay were used tomato plants with approximately 30 days of age grown in disposable cups of 500 ml. Initially, these plants were sprayed with 6 ml of each solution tested, according to previous bioassay. After drying the solutions applied, the plants were distributed randomly into cages (70 × 35 × 35 cm), four plants per cage in a total of 10 replicates (cages). Then, in each cage 10 adult couples were released and after 48 hours the eggs were counted in each treatment.

2.8 Statistical Analysis

The normality and homoscedasticity of the residues were checked with the Shapiro-Wilk and Bartlett tests, respectively. The lethal concentrations LC50 and LC90 were calculated by Probit analysis (Finney, 1971).

Survival of the caterpillars over time was subjected to survival analysis using the Weibull model. Survival curves were then estimated and compared using contrast analysis ($P < 0.05$).

The number of eggs (*i.e.*, hatchability and/or oviposition preference) were submitted to deviance analysis and generalized linear models (GLM) with adjustment for Poisson error distribution, for which the log link was used. When necessary the treatments were compared by contrast analysis ($P < 0.05$), always using software R (version 3.1.1) integrated with the stats, survival and car packages (R-CoreTeam, 2017).

3. Results and Discussion

3.1 Toxic Effects of A. mucosa on T. absoluta Caterpillars

The lethal concentrations estimated for the crude extract of *A. mucosa* in first instar caterpillars of the tomato leaf miner are presented in Table 1.

Table 1. Lethal Concentrations (50 and 90) of crude extract of *A. mucosa* for *T. absoluta* in 24 hours

N[1]	Inclination (±SEM[2])	LC50 (CI95[3])	LC90 (CI95)	DF[4]	p-value
20	3.9^{-3}±8.9^{-4}	21.13[5] (12.01-30.25)	62.62 (49.36-75.88)	6	96.4^{-4}

Note. [1]Number of insects used; [2]Standard error of the mean; [3]Confidence interval of 95%; [4]Degrees of freedom; [5] LC values are in parts per million (ppm).

It was observed that the survival curves ($\chi^2 = 253.85$, df = 4 and $P < 0.0001$) differed, since the crude extract of *A. mucosa* significantly reduced the survival of *T. absoluta* caterpillars in all treatments in relation to controls water and methanol.

The LC90 of *A. mucosa* provided high lethality for *T. absoluta* caterpillars on the first day of evaluation, not differing from the positive control with chlorfenapyr insecticide. The LC50 obtained an intermediate result, since it statistically differs from the other treatments (Figure 1):

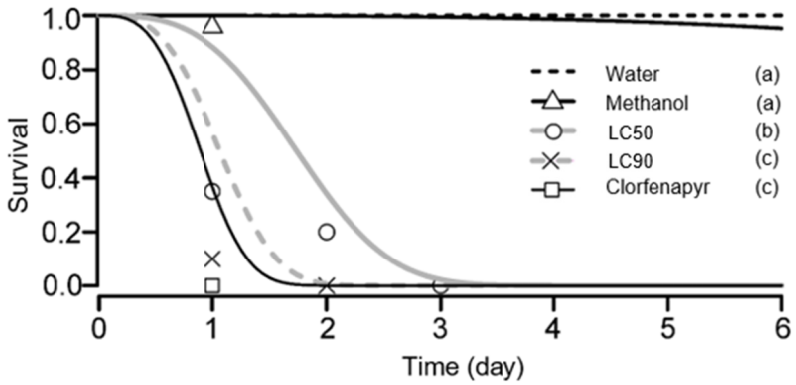

Figure 1. Survival curves for *T. absoluta* caterpillars in tomato leaflets treated with LC90 and LC50 from *Annona mucosa* crude extract, chlorfenapyr, methanol and water

It was noteworthy that the extract in the lethal concentration for 90% and clorfenapyr caused mortality of all caterpillars only by contact with the contaminated surface, because the caterpillars did not even feed on the leaves.

It was observed, therefore, that the survival curve of *T. absoluta* obtained by the LC50 exposure of *A. mucosa* decreased gradually, since up to the fourth day 100% of mortality was obtained (Figure 1), and during this period time the surviving individuals presented the following behaviors: 1) feeding followed by death, possibly associated with the action of ingestion of the extract and 2) low mobility and inhibition of feeding, indicating a probable action by contact.

Evidence of action by ingestion and contact was also observed in the histological analyzes of the larvae treated with the extract. In the control treatment, the caterpillars had the midgut externally composed of two layers of striated muscle tissue, one of which muscle arranged circularly, and the other with the muscles arranged longitudinally and with spaced bundles (Figure 2A). Internally, showed simple epithelial tissue composed of columnar cells with innumerable apical microvilli and central spherical nuclei, as well as regenerative cells and goblet cells (Figure 2B). In addition, showed well-structured tegument tissue with juxtaposed cells (Figure 2C).

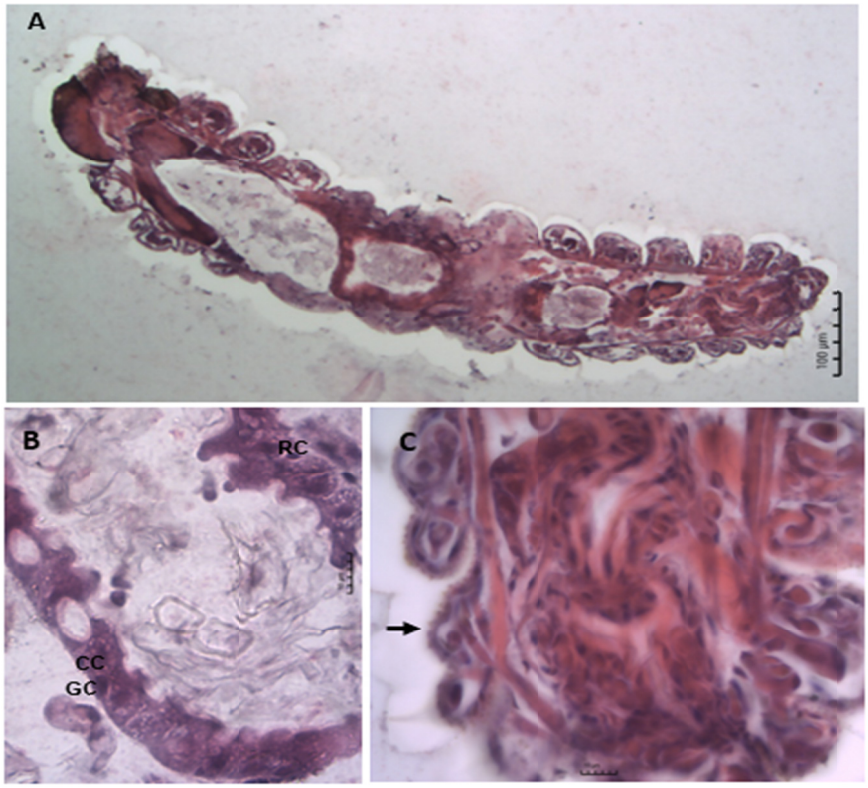

Figure 2. Photomicrograph of control caterpillars of *Tuta absoluta* stained by HE. A) Overview of longitudinal section. B) Detail of the gut with columnar cells (CC), goblet cells (GC) and regenerative cell (RC). C) Detail of the posterior portion. Note the integument with juxtaposed epithelial cells (arrow)

However, the caterpillars treated with LC50 presented structural disorganization of the epithelium od midgut with presence of vacuoles in columns cells and absence of goblet and regenerative cells (Figure 3A). In this treatment there were also ruptures of the tegument cell layers (Figure 3B).

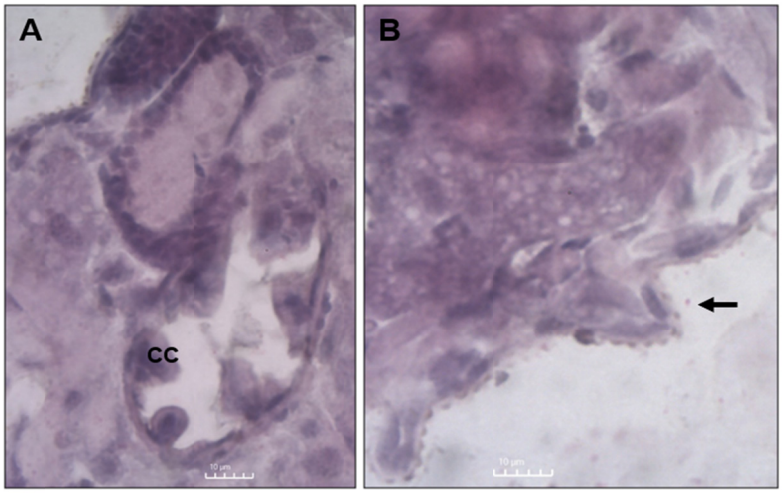

Figure 3. Photomicrograph of the gut of *Tuta absoluta* caterpillars submitted to LC50 treatment of *Annona mucosa*. A) Note gut with few apparently unstructured and nonuniform columnar cells (CC). B) Note the integument with apparently affected cells (arrow) and cells with great cytoplasmic vacuolization

Changes in tissue morphology similar to those analyzed in *T. absoluta* have already been observed in other insects, when using secondary plant metabolites (Correia et al., 2009; Costa et al., 2014; Barreto, Cavasin, Garcia, & Silva, 2006). For this purpose, it is believed that there is an important relationship between the chemical structure of the extract components and the biological activity of these compounds (E. Kim, H. Kim, Choi, & Ahn, 2003). In general, the greater the lipophilicity of the compound the greater the penetration into the integument of the insect. The rupture of the integumentary tissue of the treated caterpillars may be due to the lipophilic characteristic of the extract used and this shows that it has a mode of action per contact.

The modes of action of *A. mucosa* have already been attested in researches with species of insects of agricultural importance. In the brown stink bug *Euschistus heros* Fabricius (Hemiptera: Pentatomidae) and stink bug *Tibraca limbativentris* Stal (Hemiptera: Pentatomidae) the extract exhibits contact action (Turchen, Hunhoff, Paulo, Souza, & Pereira, 2016; Krinski & Massaroli, 2014), and the corn caterpillar *S. frugiperda* and maize weevil *Sitophilus zeamais* Motschulsky (Coleoptera: Curculionidae) have already been reported on ingestion (Ribeiro et al., 2016; Ribeiro et al., 2013).

The different modes of action of the *A. mucosa* extract allow for the expansion in its use as well as can provide greater field control efficiency. The fact that the extract reaches the insect in different ways can be attributed to the synergistic interaction of the various acetogenins and other molecules of the extract (Ribeiro et al., 2014).

3.2 Toxic Effect on T. absoluta Eggs

The extract of *A. mucosa* and the chlorfenapyr insecticide showed ovicidal activity on tomato leaf miner eggs, which statistically deferred from water and methanol controls (Figure 4):

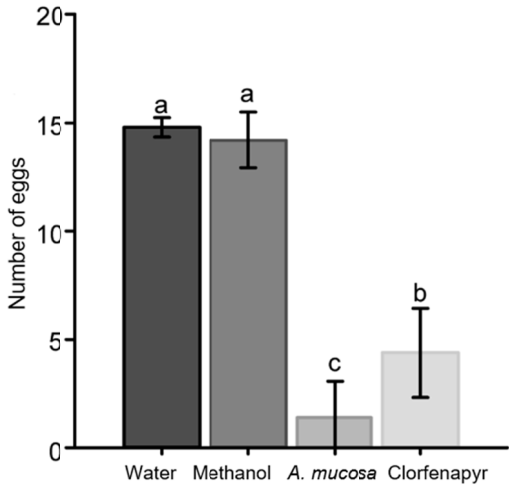

Figure 4. Average numbers of tomato leaf miner eggs hatched after treatment with crude extract of *A. mucosa,* chlorfenapyr and water and methanol controls

Until the third day of embryonic development, the eggs treated with *A. mucosa* and chlorfenapyr showed the same characteristics of the eggs from the control treatments. However, on the fourth day of observation the eggs treated with extract showed a stoppage of the embryonic development during the formation of the cephalic capsule. Already eggs sprayed with chlorfenapyr completed the embryonic development, but showed a low hatch rate in relation to the negative controls (water and methanol).

The reduction of hatchability and high inviability of the treated eggs (91%) probably occurred due to the penetration of the *A. mucosa* extract through the egg chorion. The accumulation of the extract in the inner layers exerted toxic action and caused paralysis in the development of the embryo, as observed by Salkeld and Potter (1953) in eggs of different insects. The chlorfenapyr insecticide did not interfere in embryogenesis, however, the embryos formed failed to break the egg chorion, as observed by Silva, Schneider, and Conte. (2013).

In both treatments (extract and chlorfenapyr) the few caterpillars that managed to hatch, died soon after and did not provoke any injury to the leaves. The sprayed products were probably retained on the surface of the chorion, and the neonate caterpillars, when they opened the exit orifice, came in contact with the product and died (Tomé, Cordeiro, Rosado, & Guedes, 2013).

Therefore, it was evidenced that the active compounds of the extract of *A. mucosa* act in the embryogenesis and impregnate the embryonic development, thus avoiding the damages caused by the larval stages.

3.3 Oviposition Repellency of T. absoluta

In the bioassay with a chance of choice, a significant change was observed in the oviposition of *T. absoluta* females among the treatments to which they were exposed (χ^2 = 316.02; gl. = 3; $P < 0.001$). Plants treated with *A. mucosa* extract, followed by plants treated with chlorfenapyr (insecticide) were the least preferred as oviposition site (Figure 5):

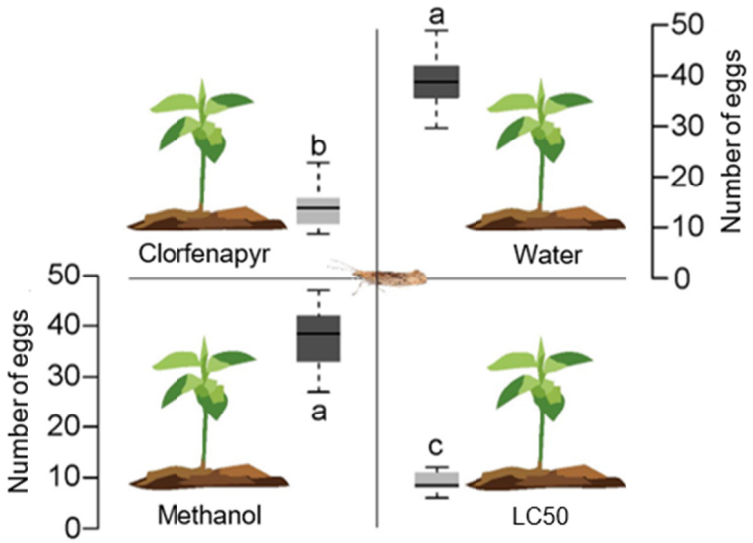

Figure 5. Number of eggs plant of the tomato leaf miner under test on oviposition with possibility of choice exposed to the treatment of the crude extract of *A. mucosa*, chlorfenapyr, and water and methanol controls

In general, there was a change in the oviposition behavior of the females, since in the water and methanol controls a greater number of eggs occurred, and the preference of the moths was oviposition in the leaf veins. Already in the treatment corresponding to the extract of *A. mucosa* and in the positive control chlorfenapyr the eggs were placed in smaller quantity and in a more dispersed way.

The reduction in oviposition probably occurred due to the perception of females when touching the surface of the impregnated leaf (extract/insecticide), with tarsi and antennas, which led to changes in oviposition behavior (Kumari & Kaushik, 2016). The efficiency of the crude extract of *A. mucosa* in reducing oviposition has already been observed in *P. xylostella* with a deterrence of more than 70% the highest concentrations (Souza, Hoffmann, Massaroli, & Pereira, 2015).

4. Conclusion

The extract of *A. mucosa* showed a toxic effect on caterpillars at low concentrations, resulting in both ingestion and contact modes of action. The extract interfered in the embryogenesis and oviposition behavior of *T. absoluta*.

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