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Cytotoxicity of *Piper aduncum* (Piperaceae) essential oil in brown stink bug *Euschistus heros* (Heteroptera: Pentatomidae)

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Abstract

Euschistus heros (F.) (Hemiptera: Pentatomidae) is a soybean pest in Brazil, controlled with synthetic chemical insecticides, which may be harmful to the environment and humans, as well as to select pest resistant strains. The research for new pest control strategies such as the use of plant essential oils has been increased due to the selectivity and biodegradation of these molecules. The objective was to evaluate the cytological changes in the salivary glands, fat body and midgut of *E. heros* exposed to different concentrations of essential oil of *Piper aduncum* L. (Piperales: Piperaceae), which the main compounds were identified as myristicin 30.03%, aromadendrene 9.20%, dillapiole 8.43%, α-serinene 7.31%, tridecane 6.26%, γ-elemene 4.58% and o-cymene 4.20%. The essential oil of *P. aduncum* was toxic for *E. heros* with $LD_{50} = 36.23$ mg per insect and $LD_{90} = 50.42$ mg per insect. Cytological changes such as tissue disruption, increase in mitochondria population, and glycogen and lipid depletion occur in the fat body cells, whereas salivary glands and midgut are not affected by this essential oil. Results suggest that *P. aduncum* essential oil causes fat body cellular stress, which may compromise some physiological processes for the insect survival.

Keywords Botanical insecticide · Fat body · Midgut · Pest control · Salivary glands · Toxicity

Introduction

The brown stink bug, *Euschistus heros* (F.) (Heteroptera: Pentatomidae) is a soybean pest in Brazil which is controlled with thiamethoxam, lambda-cyhalothrin (Hegeto et al. 2015), acephate, bifentrin, and imidachloprid (Ribeiro et al. 2016). However, the use insecticides have been selected pest resistant populations (Sosa-Gomez et al. 2001; Sosa-Gomez and Silva 2010). Synthetic insecticides cause side effects such as the pest resurgence, emergence of new pests and the reduction of beneficial insect fauna (Hemingway et al. 2002). Thus, it is necessary to research new selective and biodegradable molecules (Simmonds et al. 2002; Youssef et al. 2004).

Botanical insecticides have been used in integrated pest management (IPM) because they are safe for human health and less persistent in the environment than synthetic chemicals (Isman 2000; Martínez et al. 2015). The bioactivity of these compounds on pests includes repellency, oviposition and antifeeding, growth regulators and toxicity to larvae and adults with low pollution and rapid environmental degradation (Bakkali et al. 2008; Costa et al. 2017a; Plata-Rueda et al. 2017). Histological and cytotoxic effects of botanical insecticides are poorly studied, but they cause changes in insects organs such as midgut of *Ceraeochrysa claveri* (Neuroptera: Chrysopidae) exposed to neem essential oil (Scudeler et al. 2016), squamocin on midgut and anal papilla of *Aedes aegypti* (Diptera: Culicidae) (Costa et al. 2017a; 2017b), and midgut of *Anticarsia gemmatalis* (Lepidoptera: Noctuidae) (Fiaz et al. 2018). Also, essential oil of *Piper hispidinervum* caused damages in ovary of *Spodoptera frugiperda* (Lepidoptera: Noctuidae) (Alves

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et al. 2014), affecting the physiology, reproduction, and survivorship.

Representatives of Piperaceae produce many essential oils (Assis et al. 2013) and have been investigated for control insect pests. Essential oil of *Piper aduncum* L. (Piperales: Piperaceae) has insecticidal activity against Coleoptera (Estrela et al. 2006; Fazolin et al. 2007), Diptera (Bernard et al. 1995; Misni et al. 2011), Hemiptera (Piton et al. 2014; Volpe et al. 2015), and Hymenoptera (Souto et al. 2012). Despite the insecticidal efficacy of that essential oil for different insects, little is known about the cytotoxic effects on internal organs, such as the salivary glands, midgut, and fat body, important for insect homeostasis.

In *E. heros*, the morphology of the salivary glands, midgut, and fat body has been described (Peiffer and Felton 2014; Castellanos et al. 2017), but histological and cytotoxic effects in these organs caused by chemical and botanical insecticides are scarce.

The objective was to determinate the lethal doses (LD_{25}, LD_{50}, LD_{75}, and LD_{90}) and evaluate the cytotoxicity of *P. aduncum* essential oil in the salivary glands, midgut, and fat body of *E. heros*.

Material and methods

Insects

Adults of *E. heros* were obtained from Laboratory of Celular Ultrastructure of the Federal University of Viçosa (state of Minas Gerais, Brazil), reared at 27 ± 2 °C temperature, 70 ± 10% relative humidity, and 14:10 h (light: dark) photoperiod. The insects were maintained in plastic containers (1000 mL) covered with filter paper and with cotton balls as oviposition sites. The adults and nymphs were fed *ad libidum* with bean pods, grains of soybean, peanuts, and sunflower seeds, plus cotton moistened ball. The eggs were maintained in Petri dishes with bean pods until reach the 3rd instar. After this, the insects were transferred to plastic containers described above (Silva et al. 2008).

Essential oil

Plants of *P. aduncum* were collected in Tangará da Serra, state of Mato Grosso, Brazil (14°29' S, 57°54' W) between December 2011 and January 2012. Leaves of *P. aduncum* were dried at 37 °C for 96 h and milled using a 1 mm diameter knit mill. The powder was submitted to hydrodistillation (100 g for 3 h), with a Clevenger extractor. The sample was separated by the decantation method with a funnel in dichloromethane. The fractions were dried with anhydrous $CaCl_2$. The salt was removed by vacuum filtration and the solvent was evaporated at room temperature. The main compounds of that essential oil here used are myristicin 30.03%, aromadendrene 9.20%, dillapiole 8.43%, α-serinene 7.31%, tridecane 6.26%, γ-elemene 4.58% and o-cymene 4.20% (Turchen et al. 2016).

Toxicity test

The essential oil of *P. aduncum* was diluted in 1 mL of acetone to obtain a stock solution. Five concentrations of essential oil were prepared and used to assess insecticide toxicity and determine relevant toxicological endpoints; a dilution series of concentrations (20, 40, 60, 80 and 100 mg mL^{-1}) was used to determine concentration–mortality relationship and lethal doses (LD_{25}, LD_{50}, LD_{75}, and LD_{90}). Acetone was used as a control. Each solution (5 µL) was applied to the thorax of 50 *E. heros* adults (48 h old) using a 1–10 µL micropippette (Eppendorf®, Hamburg, Germany). Insects were placed individually in Petri dishes (90 × 15 mm) with a perforated cap for ventilation and absorbent paper, provided with bean pods, grains of soybean, peanuts, and sunflower seeds, and maintained in darkness. Three replicates of 50 insects each were used for each of the five concentrations tested and control following a completely random design. The number of dead adults in each vial was counted after 24 h. Dose–mortality data were subjected to probit analysis, generating a dose–mortality curve (Finney 1964). Toxicity data were analyzed using SAS for Windows v. 9.0. (SAS Institute 2002).

Histopathology

Adults of *E. heros* exposed for 24 h to LD_{50} of *P. aduncum* essential oil and control were cryosanesthetized at –4 °C, dissected in insects' saline solution (0.1 M NaCl + 0.1 M KH_2PO_4 + 0.1 M Na_2HPO_4) and the salivary gland, fat body and midgut transferred to Zamboni fixative solution (Stefanini et al. 1967) for 12 h at 5 °C. The samples were dehydrated in a graded ethanol series (70, 80, 90 and 95°) and embedded in historesin JB4 (Electron Microscopy Sciences, Fort Washington, PA). Sections with 3 µm thickness were stained with hematoxylin and eosin and analyzed with Olympus BX-60 light microscope (Olympus Corporation, Tokyo, Japan).

Some unstained sections were submitted to P.A.S. histochemical test for determination of glycogen and glucoconjugates. Briefly, samples were immersed in 0.4 M periodic acid for 30 min, washed in distilled water and incubated in Schiff's reagent for 1 h in the dark. After washing in sulfur water, the samples were counterstained with hematoxylin and analyzed under light microscope.

Citotoxicity

The salivary glands, midgut and fat body of *E. heros* exposed to the estimated LD_{50} lethal dose of the *P. aduncum* essential oil and control were dissected as described and transferred to 2.5% glutaraldehyde in 0.2 M sodium cacodylate buffer pH 7.2 containing 0.2 M sucrose for 4 h at room temperature. The samples were post-fixed in 1% osmium tetroxide in the same buffer for 2 h, followed by washing in the buffer and dehydration in a graded of ethanol series (70, 80, 90 and 98°). The samples were embedded in LR White resin (London Resin Company Ltd.) and sectioned with glass knives (60–70 nm thick) in Power Tome PT-X ultramicrotome (RMC Boeckeler Instruments Inc., Tucson, AZ, USA). The ultrathin sections were stained with 1% aqueous uranyl acetate and lead citrate (Reynolds 1963) and examined with Zeiss EM 109 transmission electron microscope (Carl Zeiss, Jena, Germany).

Results

Toxicity test

The dose–mortality model used was suitable ($P > 0.05$), confirming the toxicity of *P. aduncum* essential oil to *E. heros* and allowing estimates toxicological doses for subsequent use (Table 1; Fig. 1). Mortality in the control group was < 1%.

Histopathology

Salivary gland

The principal salivary gland of *E. heros* was formed by an anterior and posterior lobe separated by a hilus (H) (Fig. 2a). The anterior lobe had a single layered epithelium with cubic cells and well-developed nucleus, cytoplasm with few vacuoles and lumen with homogeneous and basophilic content (Fig. 2a). In the posterior lobe of the gland, the epithelium has flattened cells, with an elongated nucleus, cytoplasm with few vacuoles and lumen with content weakly basophilic (Fig. 2a). The principal salivary gland of *E. heros* exposed to *P. aduncum* essential oil had no histological changes (Fig. 2b). Histochemical P.A.S. test showed few glycogen storages in the salivary gland in both control and treated insects (data not shown).

Midgut

The midgut of *E. heros* had a single layered epithelium with columnar cells with well-developed apical striated border and cytoplasm with vacuoles and small granules (Fig. 2c). The nucleus was well developed and rich in decondensed chromatin (Fig. 2c). Some cell had the apical surface irregular with projections towards the lumen (Fig. 2c). The midgut epithelium of *E. heros* exposed to *P. aduncum* essential oil had no histological changes (Fig. 2d) in comparison with control ones. Histochemical P.A.S. test showed many glycogen storages in the digestive cells in both control and treated insects, so the treatment did not affected this organ (data not shown).

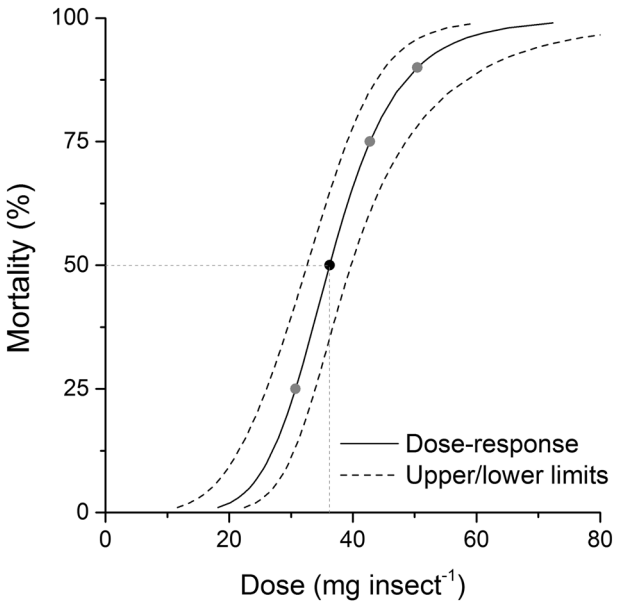
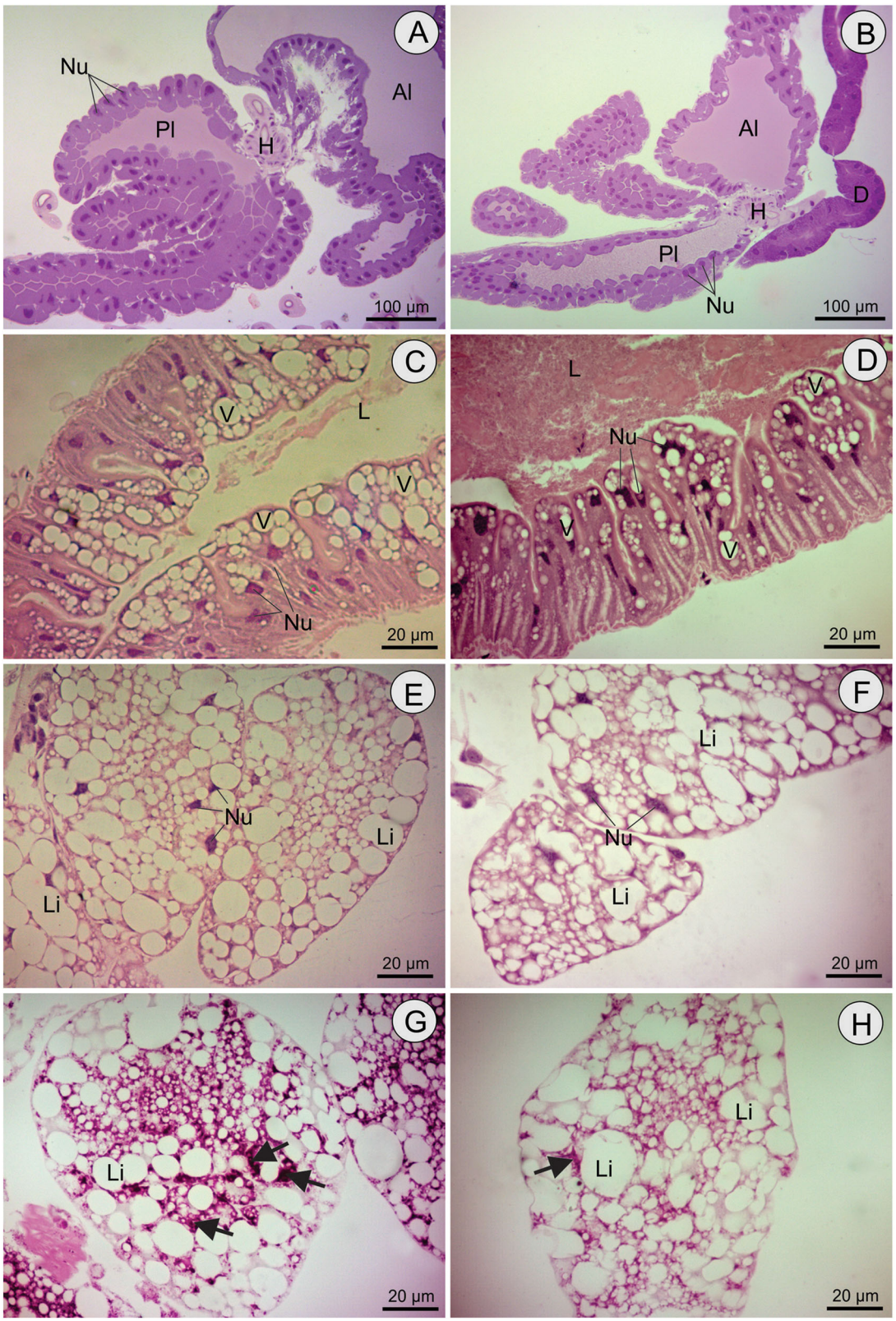


Fig. 1 Toxicity of *Piper aduncum* essential oil by contact on *Euschistus heros*. Lethal dose values (LD) were estimated based on the dose-mortality assays using Probit analyzes ($LD_{50} = 36.23$ (32.59–39.70) mg insect^{-1}, $\chi^2 = 3.55$; df = 5; $P > 0.05$). Dotted lines denote 95% confidence intervals. Black dot represents the LD_{50} selected to evaluate histological and ultrastructural changes

Table 1 Lethal doses of *P. aduncum* essential oil against *Euschistus heros* after 24 h exposure obtained from probit analysis (df = 5, Slope ± SE = 8.856 ± 1.51, intercept = 4.923)

No. Insects	Lethal doses	Estimated dose (mg per insect)	95% Confidence Interval (mg per insect)	χ^2 (*P*–value)
150	LD_{25}	30.72	26.03–33.92	3.55 (0.46)
150	LD_{50}	36.23	32.59–39.70	
150	LD_{75}	42.74	39.05–48.54	
150	LD_{90}	50.42	45.17–61.49	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◀ **Fig. 2** Light micrographs of the principal salivary glands, midgut and fat body of *Euschistus heros* exposed to *Piper aduncum* essential oil. **a** Salivary glands of control group. **b** Salivary glands of the group treated with *P. aduncum*. Epithelial cells lining the lumen of the anterior (*Al*) and posterior (*Pl*) lobes showing the spherical and central nucleus (*Nu*) beyond the uniformly stained cytoplasm separated by hilus (*H*). Duct cells (*D*) without change after treatment. Cells showing cytoplasm more acidophilous than secretory epithelial cells and spherical central nucleus. **c** Midgut control group. **d** Midgut of the group treated with *P. aduncum* essential oil. The epithelium lining the gut lumen (*L*) is columnar in shape with nuclei (*Nu*) at different heights. The cytoplasm of epithelial cells has many vacuoles (*V*) of different sizes. **e** Fat body of insects of the control group presenting trophocytes in the cytoplasm filled by lipids droplets (*Li*) and peripheral nuclei (*Nu*) **f** Fat body group treated with *P. aduncum* essential oil with peripheral nuclei (*Nu*) and some reduction in diameter of lipid droplet *(Li)*. **g–h**. Fat body submitted to PAS technique. In control insects **g** showing trophocytes with glycogen granules accumulated in the cytoplasm (arrows) and lipid droplets (*Li*). Fat body treated group **h** showing reduction of glycogen granules (arrow) and disrupting of lipid droplets (*Li*)

Fat body

The *E. heros* fat body had cells forming small nodules in the hemocele associated with trachea (Fig. 2e). The fat body cells had spherical nucleus and cytoplasm with large lipids droplets (Li) (Fig. 2e) and glycogen storage (arrows). In the insects treated with *P. aduncum* essential oil fat body cells were similar to the control (Fig. 2f), except by a strong decrease in glycogen and lipid content (Fig. 2g, h).

Citotoxicity

Likely histological analyses, the salivary glands and midgut cells of *E. heros* had no differences in relation to the control group. So, they are not described herein. However, some effects were detected in the fat body cells.

In the control insects, fat body cells had nucleus rich in decondensed chromatin with some cloths of condensed chromatin (Fig. 3a, b). The cytoplasm had some mitochondria, rough endoplasmic reticulum (Fig. 3b, c) and showed lipid droplets (Fig. 3a–c). In *E. heros* exposed to *P. aduncum* essential oil, fat body cells showed enlarged intercellular spaces between causing tissue disruption (Fig. 3d). The nucleus showed increase in chromatin condensation (Fig. 3e) and the amount of lipid droplets decreased (Fig. 3d) whereas mitochondria increased in number (Fig. 3f).

Discussion

The toxicity of *P. aduncum* essential oil to the stink bug, *E. heros* was determined from tests performed under laboratory conditions. The *P. aduncum* essential oil is toxic to adult *E. heros* and had a strong effect upon topical application ($LD_{50} = 36.23$ mg per insect and $LD_{90} = 50.42$ mg per insect). The susceptibility of other insect pests such as *Chrysodeixis includens* (Lepidoptera: Noctuidae), *Diaphorina citri* (Hemiptera: Psyllidae), and *Tibraca limbativentris* (Heteroptera: Pentatomidae) may vary depending on the method of exposure to *P. aduncum* essential oil (contact or ingestion) (Volpe et al. 2015; Krinski and Foerster 2016; Sanini et al. 2017). The effectiveness of *P. aduncum* as insecticides has been associated with high concentrations of piperamides that act as neurotoxins (Scott et al. 2008) affecting axonal sodium channels, a similar effect caused by pyrethroid insecticides (Miyakado et al. 1979; Elliott et al. 1986; Lees and Burt 1988). In addition, *P. aduncum* essential oil has been reported to be selective for parasitoid wasps *Telenomus podisi* (Hymenoptera: Platygastridae) and *Trissolcus urichi* (Hymenoptera: Platygastridae) used in biological control of *E. heros* (Turchen et al. 2016). In general, topical application of *P. aduncum* essential oil at different doses and small volumes was sufficient to cause toxicity in *E. heros*, showing an insecticidal potential of this essential oil to pest control.

The cytological changes of *P. aduncum* essential oil in the fat body cells of *E. heros* may be due to the toxicity of myristicin and dillapiole, the main compounds in this essential oil (Turchen et al. 2016). Myristicin has been reported to be toxic against *Spilarctia obliqua* (Lepidoptera: Erebidae) larvae (Srivastava et al. 2001), *Drosophila melanogaster* (Diptera: Drosophilidae) (Lichtenstein and Casida 1963), whereas dillapiole is toxic for *Anopheles marajoara* and *Aedes aegypti* (Diptera: Culicidae) larvae (Almeida et al. 2009). In the fat body cells of *E. heros, P. aduncum* essential oil decreases the glycogen and lipid reserves. The fat body has been reported as non-target organ affected by insecticides with decrease in the stored reserves (Nath 2003; Guedes et al. 2006).

Fat body cells are important for lipid and carbohydrates storage (Martins et al. 2011a). Decrease in these compounds in *E. heros* exposed to *P. aduncum* essential oil, suggests use of these energy molecules for some detoxification. Glycogen serves as nutritional reserve and it is first consumed by the cell as energy resource (Alberts et al. 2015). In contrast, lipids may function both as reserves and signaling molecules and their decrease in the fat body cells may indicate both energy conversion (lipids have high caloric content) and signaling for detoxification cascade, since fat body is hypothesized to be a dynamic organ playing roles as signaling, affecting many insect physiological processes (Arrese and Soulages 2010). Thus, *P. aduncum* essential oil causes cytological and physiological changes in the *E. heros* fat body with detrimental effects on growth and reproduction as alterations in the synthesis and secretion of proteins of the oocytes (Attardo et al. 2005), during the embryonic phase (Lucas et al. 2015).

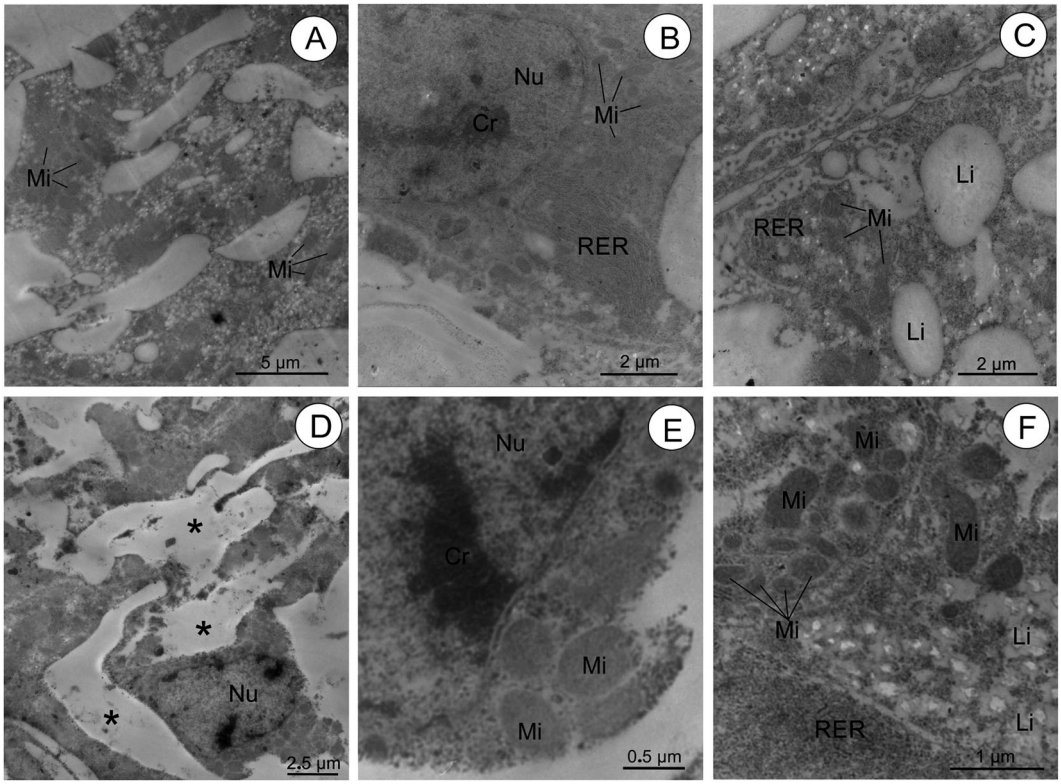

Fig. 3 Transmition electron micrographs of the *Euschistus heros* fat body exposed to *Piper aduncum* essential oil. **a–c** Control group. **a** Overview of trophocytes. **b** Trophocytes showing spherical nucleus (*Nu*), condensed chromatin (*Cr*) and cytoplasm containing mitochondria (*Mi*) and rough endoplasmic reticulum (*RER*). **c**. Detail of the trophocytes cytoplasm containing large lipid droplets (*Li*). **d–f** Group treated with *P. aduncum* essential oil. **d** Overview of treated trophocytes. Note tissue disruption between cells due to increase in the intercellular space (*). **e** Perinuclear region showing nucleus (*Nu*) with greater area of condensed chromatin (*Cr*) and cytoplasm with mitochondria (*Mi*). **f** Detail of trophocyte cytoplasm rich in rough endoplasmic reticulum (*RER*), increased mitochondria (*Mi*) population and reduction of lipid content (*Li*) compared to the control group

Another change in the fat body cells of *E. heros* exposed to *P. aduncum* essential oil is the increase in the mitochondrial population. A similar finding has been reported in neurosecretory cells of the locust *Schistocerca gregaria* exposed to insecticide lindane (Normann and Samaranayaka-Ramasamy 1977) and in rat and human nerve cells exposed to rotenone (Barsoum et al. 2006). The increase in the mitochondrial population associated with exploit of glycogen and lipid reserves in the fat body cells of *E. heros*, suggests a high energy conversion, perhaps to enhance the detoxification of the *P. aduncum* essential oil compounds in these organs, which play a central role in xenobiotic metabolism (Guedes et al. 2006; Martins et al. 2011b). However, cannot be ruled out that increase in the amount of mitochondria may result in some deleterious effects in the fat body cells, because this organelle is duplicated by growth and fission of existent mitochondria and high mitochondrial fission rate has been reported as a mitopathology followed by cell death (Barsoum et al. 2006; Knott and Bossy-Wetzel 2008).

Conclusion

The insecticide potential of *P. aduncum* essential oil against *E. heros* was studied. Our findings show that *P. aduncum* is toxic and cytotoxic in the fat body cells, decreasing glycogen and lipid reserves and increasing the mitochondrial population, suggesting that this essential oil may efficiently control to *E. heros*. Thus, *P. aduncum* essential oil exhibits lethal and sublethal effects on *E. heros* and can be an alternative to synthetic insecticides aiding in eventual insecticide resistance management efforts. Also, *P. aduncum* essential oil may be a powerful tool to be used in integrated pest management programs due to their safety to non-target organisms such as parasitoid wasps, health hazard, and to the environment, meeting the criteria for reduced-risk pesticides. Our findings show that *P. aduncum* essential oil is a potential source of insecticide. For this essential oil, constituents are toxic ones to insects and futures studies can involve the mode of action or other cell physiological effects and warrants further research.

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Compliance with ethical standards

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

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