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Insecticidal activity of *Pilocarpus spicatus* Saint-Hilaire (Rutaceae) essential oil against the crop pest *Dysdercus peruvianus* (Guérin-Méneville, 1831) and *Oncopeltus fasciatus* (Dallas, 1852)

Atividade inseticida do óleo essencial de *Pilocarpus spicatus* Saint-Hilaire (Rutaceae) contra a praga agrícola *Dysdercus peruvianus* (Guérin-Méneville, 1831) e *Oncopeltus fasciatus* (Dallas, 1852)

Actividad insecticida del aceite esencial de *Pilocarpus spicatus* Saint-Hilaire (Rutaceae) contra la plaga agrícola *Dysdercus peruvianus* (Guérin-Méneville, 1831) y *Oncopeltus fasciatus* (Dallas, 1852)

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Raul Apolinário
ORCID: https://orcid.org/0000-0002-3026-4619
Universidade Federal Fluminense, Brazil
E-mail: apolinario_raul@yahoo.com
Jeane Nogueira
ORCID: https://orcid.org/0000-0003-3613-4935
Universidade Federal Fluminense, Brazil
E-mail: jeanogueira@hotmail.com
Meiriellem G. da Silveira Costa
ORCID: https://orcid.org/0000-0002-5974-0287
Universidade Federal Fluminense, Brazil
E-mail: mei.ellen@hotmail.com
Jacenir Santos-Mallet
ORCID: https://orcid.org/0000-0003-4728-7638
Fundação Oswaldo Cruz, Brazil
Universidade Iguaçu, Brazil
E-mail: jacenir@ioc.fiocruz.br
Marcelo Guerra Santos
ORCID: https://orcid.org/0000-0002-0680-4566
Universidade do Estado do Rio de Janeiro, Brazil
E-mail: marceloguerrasantos@gmail.com

Patrícia Azambuja
ORCID: https://orcid.org/0000-0002-1405-8743
Universidade Federal Fluminense, Brazil
Instituto Nacional de Entomologia Molecular, Brazil
E-mail: azambuja.p@gmail.com
Cícero Brasileiro Mello
ORCID: https://orcid.org/0000-0002-0092-6829
Universidade Federal Fluminense, Brazil
Instituto Nacional de Entomologia Molecular, Brazil
E-mail: cicerobrasileiro@id.uff.br
Marcelo Salabert Gonzalez
ORCID: https://orcid.org/0000-0002-5442-6440
Universidade Federal Fluminense, Brazil
Instituto Nacional de Entomologia Molecular, Brazil
Universidade Federal do Rio de Janeiro, Brazil
E-mail: msgonzalez@id.uff.br
Leandro Rocha
ORCID: https://orcid.org/0000-0003-0484-1918
Universidade Federal Fluminense, Brazil
E-mail: lean.machado@gmail.com
Maria Denise Feder
ORCID: https://orcid.org/0000-0002-6883-0583
Universidade Federal Fluminense, Brazil
Instituto Nacional de Entomologia Molecular, Brazil
Email: mdfeder@id.uff.br

Abstract

Studies were carried out to evaluate the insecticidal activity of *Pilocarpus spicatus* Saint-Hilaire (Rutaceae) essential oil (EO) on the development of the Hemiptera *Dysdercus peruvianus* (Guérin-Méneville, 1831) and *Oncopeltus fasciatus* (Dallas, 1852). Gas Chromatography/Mass spectrometry analysis revealed a chemical composition with sabinene (32.27%) and sylvestrene (27.26%) as major constituents. Topical and continuous treatment with the pure EO induced 100% of mortality while serial dilutions of the EO induced different levels of lethality in a dose response manner. Median lethal dose (LD50) and lethal dose 90%

(LD90) were determined. Malformations in insects and permanent or supernumerary nymphs were often observed after treatments, and the use of scanning electron microscopy allowed the analysis of morphological changes. The different biological effects of *P. spicatus* EO point out its potential as a rich source of bioactive molecules to be used as an alternative control method against agricultural pest insects.

Keywords: Essential oil; Pest control; Insect growth regulators; *Dysdercus peruvianus; Oncopeltus fasciatus*; Green pesticide.

Resumo

Estudos foram realizados para avaliar a atividade inseticida do óleo essencial (OE) de *Pilocarpus spicatus* Saint-Hilaire (Rutaceae) sobre o desenvolvimento dos Hemiptera *Dysdercus peruvianus* (Guérin-Méneville, 1831) e *Oncopeltus fasciatus* (Dallas, 1852). A análise por cromatografia gasosa/espectrometria de massa revelou uma composição química com sabineno (32,27%) e silvestreno (27,26%) como constituintes majoritários. O tratamento tópico e contínuo com o EO puro induziu 100% de mortalidade enquanto as diluições seriadas do EO induziram diferentes níveis de letalidade em respostas de dose-dependência. Foram determinadas a dose letal mediana (DL50) e a dose letal 90% (DL90) dos experimentos. Malformações nos insetos e ninfas permanentes ou supernumerárias foram frequentemente observadas após os tratamentos, e a utilização de microscopia eletrônica de varredura permitiu a análise de alterações morfológicas. Os diferentes efeitos biológicos do EO de *P. spicatus* apontam seu potencial como uma fonte rica em moléculas bioativas a serem utilizadas como método alternativo de controle contra insetos pragas agrícola.

Palavras-chave: Óleo essencial; Controle de pragas; Reguladores de crescimento de insetos; *Dysdercus peruvianus; Oncopeltus fasciatus*; Pesticida verde.

Resumen

Se realizaron estudios para evaluar la actividad insecticida del aceite esencial (AE) de *Pilocarpus spicatus* Saint-Hilaire (Rutaceae) sobre el desarrollo de los Hemiptera *Dysdercus peruvianus* (Guérin-Méneville, 1831) y *Oncopeltus fasciatus* (Dallas, 1852). El análisis por cromatografía de gases/espectrometría de masas reveló una composición química con sabineno (32,27%) y silvestreno (27,26%) como componentes principales. El tratamiento tópico y continuo con AE puro indujo 100% de mortalidad, mientras que las diluciones seriadas del AE indujeron diferentes niveles de letalidad en respuestas de dependencia de la dosis. Se determinaron la dosis letal mediana (DL50) y la dosis letal 90% (DL90) de los

experimentos. Con frecuencia se observaron malformaciones en insectos y ninfas permanentes o supernumerarias después de los tratamientos, y el uso de microscopía electrónica de barrido permitió el análisis de cambios morfológicos. Los diferentes efectos biológicos del AE de *P. spicatus* apuntan a su potencial como una rica fuente de moléculas bioactivas para ser utilizadas como un método alternativo de control de insectos plagas agrícola.

Palabras clave: Aceite esencial; Control de plagas; Reguladores del crecimiento de insectos; *Dysdercus peruvianus; Oncopeltus fasciatus*; Pesticida verde.

1. Introduction

Due to the economic importance of insects which are considered agricultural pests, many methods based on the use of synthetic chemicals for pest control have been adopted over decades. Despite the great potential of applicability, industrial chemicals have been responsible for several toxicological issues. Environmental damage and toxicity to non-target organisms were often reported in the use of first insecticides from earlier generations (Fernandes, et al., 2013). To reduce these adverse effects, it is necessary to develop innovative control methods for the agricultural pest management. The use of natural products obtained from plants have been shown wide potential as source of biodegradable green insecticides containing substances such as terpenoids, alkaloids and phenolic compounds (Mello, et al., 2008; Chaubey, 2019). Such plant-derived products (PDPs) have been recognized by several biological activities as repellency and insect toxicity, besides other bioactive effects on invertebrates (George, et al., 2014). Among PDPs, essential oils are characterized as volatile compounds often constituted by secondary metabolites (e.g. terpenes hydrocarbons, alcohols, aldehydes, ketones, phenols, and esters) (Chaubey, 2019). Rutaceae, a plant family that produces essential oils, is represented by many species that have potential in the medical, ornament and food industry (Avancini, et al., 2003; Santos & Moreno, 2004). The essential oil of *Pilocarpus* genus represents one of the most important sources of secondary metabolites with pharmacological activity (Skorupa, et al., 1998). *Pilocarpus spicatus* Saint-Hilaire can also be recognized by the heterotypic *Pilocarpus lisboanus* Badini, being endemic in Brazil with wide distribution in the Northeast, Southeast and South regions (Flora do Brasil, 2020). There are reports on the different types of biological activities of *P. spicatus* EO, including bactericidal activity (Santos, et al., 1997; Oliveira, et al., 2010), insecticidal activity (Mello, et al., 2007), immunomodulation responses (Costa, et al., 2010), antiparasitic activity on

Trypanosoma cruzi (Mafezoli, et al., 2000) and responses in edema (Silva & Rao, 1992). To comparison, we used as biological model two hemimetabolous and phytophagous insects. *Dysdercus peruvianus* (Guérin-Méneville, 1831) (Hemiptera: Pyrrhocoridae) has a great economic importance, being considered as a pest that causes damage to cotton. *Oncopeltus fasciatus* (Dallas, 1852) (Hemiptera: Lygaeidae) is not considered a pest itself but as a suitable model to insect physiological studies. Our data indicate that *P. spicatus* EO acts as a potent growth inhibitor of *O. fasciatus* and *D. peruvianus* and has the potential to be used in integrated and environmentally friendly control programs against agricultural pest insects.

2. Methodology

Botanical identification

Samples of *P. spicatus* were collected at Jurubatiba Sandbank National Park, RJ, Brazil, under the authorization no. 13659-2 of the Ministry of the Environment (Ministério do Meio Ambiente - MMA) and Chico Mendes Institute for Biodiversity Conservation (ICMBio). The material was also registered in Biodiversity Authorization and Information System (SISBIO) by the code AOD648D. The specimens were identified by Dr. Marcelo Guerra Santos (State University of Rio de Janeiro - UERJ, RJ, Brazil). Voucher specimens under reference M.G. Santos (RFFP 1824) were deposited at the Faculdade de Formação de Professores Herbarium (UERJ, RJ, Brazil).

Essential oil extraction

Leaves of the three collected specimens of *P. spicatus* were turbolized with distilled water. Then the material was placed in a 5 L bottom flask and submitted to hydrodistillation for 4 h in a Clevenger-type apparatus (Gottlieb & Magalhães, 1960). The EO was obtained and stored at 4 °C for further analysis.

Gas chromatography/Mass spectrometry analysis

The EO from *P. spicatus* leaves was analyzed by a GC/MS-QP5000 (Shimadzu) gas chromatograph equipped with a mass spectrometer using mass detector with electron impact ionization (70 eV). One milligram of each sample, dissolved in CH_2Cl_2 (1:100 mg/µL), was

injected (1 µL) separately into a RTX-5 column (0.25 mm; 30 m; 0.25 µm) (Oliveira, et al., 2010). The samples were subjected to the following chromatographic conditions: injection of 260 °C; detector temperature of 290 °C; drag gas: helium with 1 mL/min flow, split injection mode with 1:40 ratio. The oven temperature was initially maintained at 60 °C and then gradually raised to 290 °C using 5 °C/min heating ramp (Tietbohl, et al., 2012; Senatore, et al., 2013). The Retention Index (RI) of substances was calculated by interpolating the retention time of a mixture of aliphatic carbon pattern (C9-C30) (Sigma ®) analyzed under the same conditions as the samples. The substances were identified by comparison of Retention Index (RI) and mass spectrum (MS) described in the literature (Adams, 2007). The relative quantitative analysis of the substances present in the EO was performed by the method of normalizing the areas of chromatogram signals, obtained after analysis by gas chromatography coupled to flame ionization detector (GC/FID), using the same conditions described above for GC/MS.

Insect and biological assays

The insect colonies were kept at 25 °C temperature and relative humidity of 60% (Fernandes, et al., 2013; Gonzalez, et al., 2014) and are registered in Brazilian National Management System of Genetic Heritage and Associated Traditional Knowledge (SISGEN) under the number AOE95C4. Fourth-instar nymphs of *D. peruvianus* and *O. fasciatus* were chosen randomly for topical and continuous treatments to evaluate the developmental process to fifth-instar (molt) and subsequently to adult (metamorphosis). Initially, for preparation of the solution, 1 mL of EO was weighed, corresponding to 1.2 g. The topical treatment was performed using 1 µL of the pure EO applying on the dorsal cuticle of the insects (Fernandes, et al., 2013), corresponding to 1.2 mg EO. Serial dilutions were made with acetone to obtain oil concentrations of 0.6 mg/mL, 0.3 mg/mL, 0.15 mg/mL, 0.075 mg/mL and 0.0375 mg/mL. Insects in the solvent control group received topical application of 1µL of acetone while the untreated control group did not receive any manipulation. For continuous treatment, pure EO or its dilutions were applied on a filter paper placed at the bottom of Petri dishes **(**diameter of 9 cm). The filter paper (63.6 cm^2) was completely covered with 22 µL of the sample, evenly distributed over the paper with 11 aliquots of 2 µL each. Considering that 1 µL of OE corresponding to 1.2 mg, the pure EO assays were made with 415 $\mu g/cm^2$ on filter paper. Therefore, concentrations of EO containing 207 $\mu g/cm^2$, 104 $\mu g/cm^2$, 52 $\mu g/cm^2$, 26 $\mu g/cm^2$ and 13 $\mu g/cm^2$ were obtained. The nymphs were placed on the treated surfaces after 3

min for solvent evaporation and Petri dishes were then cover-up. Insect control groups were placed in untreated filter paper or filter paper treated with pure acetone (22 µL). The biological evaluation of the different treatments was undertaken during the entire time required for development from the fourth-instar nymphs to the adult stage. Parameters recorded were mortality, observations of molting and metamorphosis, and malformations. Values of Median Lethal Dose (LD50) and Lethal Dose 90% (LD90) were calculated based on the last days of treatments for both insects. All experiments were repeated three times with triplicates of 30 insects (n = 30) for topical treatment and 15 insects for continuous treatment (n = 15) and observed from one day after treatment of fourth-instar nymphs until the end of the assays (22 days for treatments with *D. peruvianus*, 30 days for treatments with *O. fasciatus*).

Scanning electron microscopy

Control and treated insects were analyzed by scanning electron microscopy. The specimens were fixed with 2.5% (v/v) glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2). After fixation, three successive washes were performed with 0.1 M sodium cacodylate buffer (pH 7.2), corresponding to 10 minutes each. Subsequently, the samples were dehydrated by incubation with ethanol (EtOH) at the concentrations of 7.5%, 15%, 30%, 50%, 70% and 90% for about 10 minutes each and finally three times of incubation of 100% EtOH. The materials were taken to dry at the critical point of CO_2 in the Autosamdri R-815 equipment and later directed to a stage of positioning in stubs which were used for metallization and microscopy processing. Insect samples of fourth and fifth-instar were fixed onto the stubs by double-sided tape, and adults were fixed by silver glue. The material was metallized in the Denton Vacuum Desk IV equipment and analyzed by the JEOL JSM-6390 LV scanning electron microscope.

Data analysis

The results were analyzed using ANOVA and Tukey's test on Graphpad Prism version 7.04, which was also used to elaborate the graphs (Fernandes, et al., 2013; Gonzalez, et al., 2014). The mortality data were expressed in mean ± standard deviation (SD). Differences between control groups and treated insects were considered statistically significant at $p < 0.05$ (Armitage, et al., 2002). For the calculation of LD50 and LD90 the Statgraphics Centurion

XV version 15.1.02 program was used, and the data used are expressing the minimum and maximum confidence limit 95%.

3. Results

The analysis of the chemical composition using gas chromatography/mass spectrometry showed 25 compounds identified in *P. spicatus* EO. The EO was composed by sesquiterpenes (22.74%) and monoterpenes (69.41%), with sabinene (32.27%) and sylvestrene (27.26%) as major constituents (Table 1).

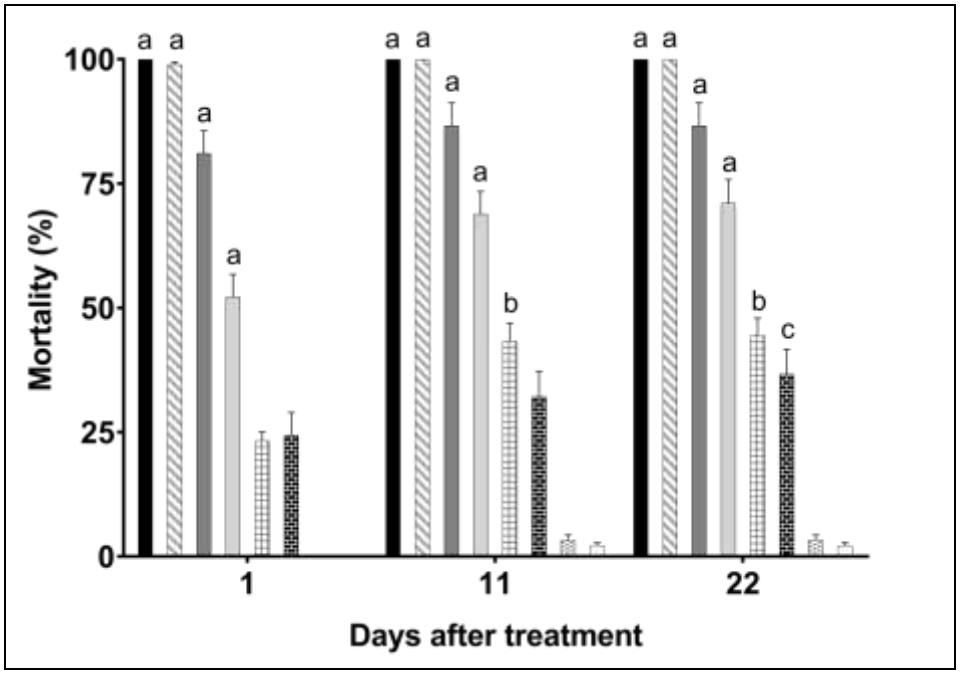
Table 1. Gas chromatography/Mass spectrometry showing the chemical composition of *Pilocarpus spicatus* essential oil. a – Retention time (minutes); b – Literature Retention Index (Adams, 2007); c – Experimental Retention Index calculated using standard C7-C30 n-alkanes.

RT[a]	Literature RI[b]	Experimental RI[c]	Constituent	Relative Abundance (%)
4.802	932	929	Pinene<α->	3.15
5.748	969	968	Sabinene	32.27
5.89	974	974	Pinene<β->	0.83
7.006	1014	1014	Terpinene<α->	1.69
7.255	1020	1021	Cymene<ρ->	0.70
7.376	1025	1025	Sylvestrene	27.26
8.342	1054	1053	Terpinene<γ->	1.09
12.984	1174	1169	Terpinen-4-ol	2.42
20.932	1350	1355	Longipinene<α->	0.33
21.451	1369	1368	Cyclosativene	1.13
21.543	1371	1370	Longicyclene	1.46
22.681	1400	1397	Longipinene<β->	2.63
23.903	1430	1428	Copaene<β->	0.88
24.849	1451	1452	Muurola-3,5-diene<trans->	0.43
24.977	1458	1456	Aromadendrene<allo->	0.55
25.183	1461	1461	Cadina-1(6),4-diene<cis->	5.93
25.701	1470	1475	Macrocarpene<α->	3.83
26.036	1487	1483	Aristolchene	0.50
26.711	1496	1500	Valencene	1.24
27.259	1514	1514	Curcumene<β->	1.58
27.941	1529	1532	Bisabolene<(E)-γ->	1.39
29.981	1594	1586	Carotol	0.37
32.327	1646	1650	Cubenol	0.49
		Total monoterpenes		69.41
		Total sesquiterpenes		22.74
		Total identified		92.15

Source: Authors.

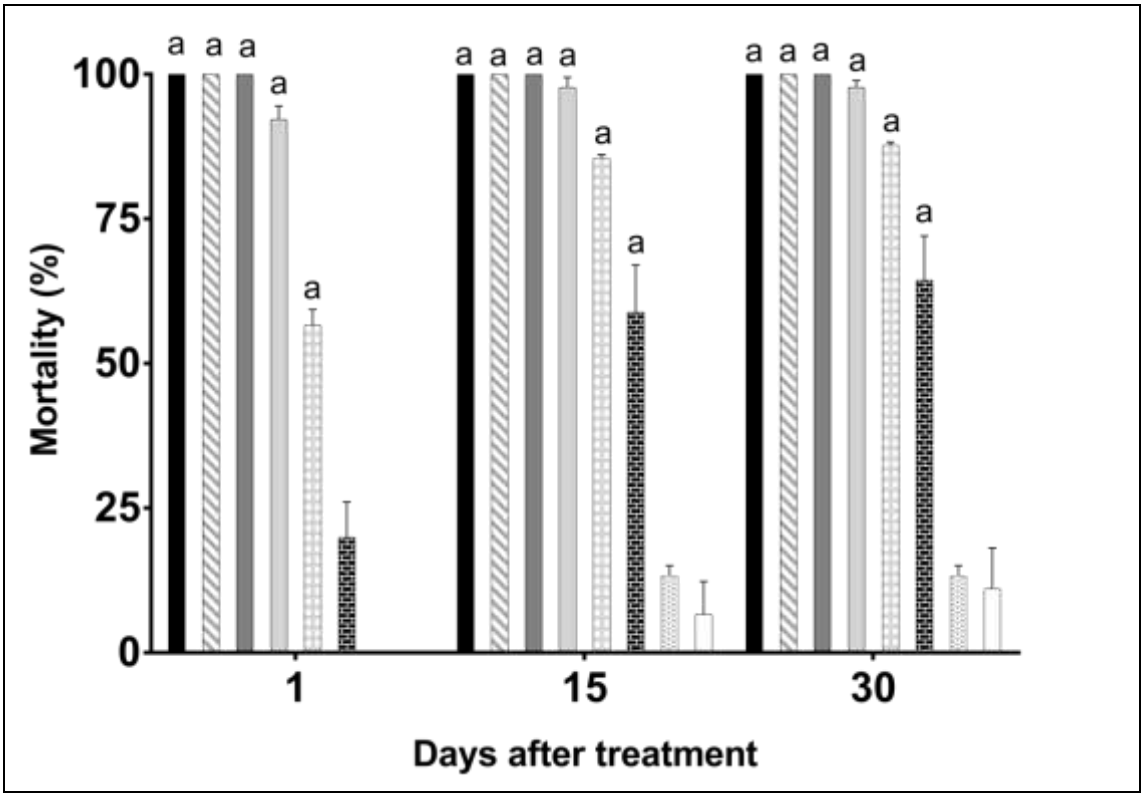
Concerning to topical application, *D. peruvianus* mortality was 3.33±1.00% ($p > 0.05$) in the solvent control group and 2.23±0.58% in the untreated control group at 22 days of treatment. *O. fasciatus* mortality was 13.33±1.73% ($p > 0.05$) in the solvent control group and 11.10±7.02% in the untreated control group at 30 days of treatment. Topical treatment with 1 µL of pure EO on *D. peruvianus* displayed 100% mortality after 24 h ($p < 0.0001$), and the same result was expressed on 0.6 mg/mL EO ($p < 0.0001$) at 11 days of treatment. In the other insect groups treated with EO dilutions of 0.3 mg/mL, 0.15 mg/mL, 0.075 mg/mL and 0.0375 mg/mL, 86.67±4.58% ($p < 0.0001$), 71.10%±4.73 ($p < 0.0001$), 44.43±3.51% ($p < 0.01$) and 36.67±5.00% ($p < 0.05$) of insects did not survived, at 22 days after treatment, respectively (Figure 1). For *O. fasciatus*, topical treatment with pure EO and dilutions of 0.6 mg/mL and 0.3 mg/mL induced 100% mortality ($p < 0.0001$) at the first day after application. Insects treated with concentrations of 0.15 mg/mL, 0.075 mg/mL and 0.0375 mg/mL displayed 97.77±1.15% ($p < 0.0001$), 87.77±0.58% ($p < 0.0001$), 64.43±7.64% ($p < 0.0001$) of mortality, at 30 days of treatment, respectively (Figure 2).

Figure 1. Percentage of mortality after topical treatment of *Dysdercus peruvianus* fourth-instar nymphs with 1 µL of pure essential oil (EO) and their different concentrations obtained from the leaves of *Pilocarpus spicatus*. ☐ Untreated control group; ▨ Solvent control group; ■ 1.2 mg (Pure EO); ▨ 0.6 mg/mL; ■ 0.3 mg/mL; ▭ 0.15 mg/mL; ▦ 0.075 mg/mL; ▦ 0.0375 mg/mL. Values are means ± SD of at least three experiments with 30 insects each. Superscript letters show statistical differences between treated and control insects: a - p <0.0001; b - p <0.01; c - p <0.05 (Tukey's test).



Source: Authors.

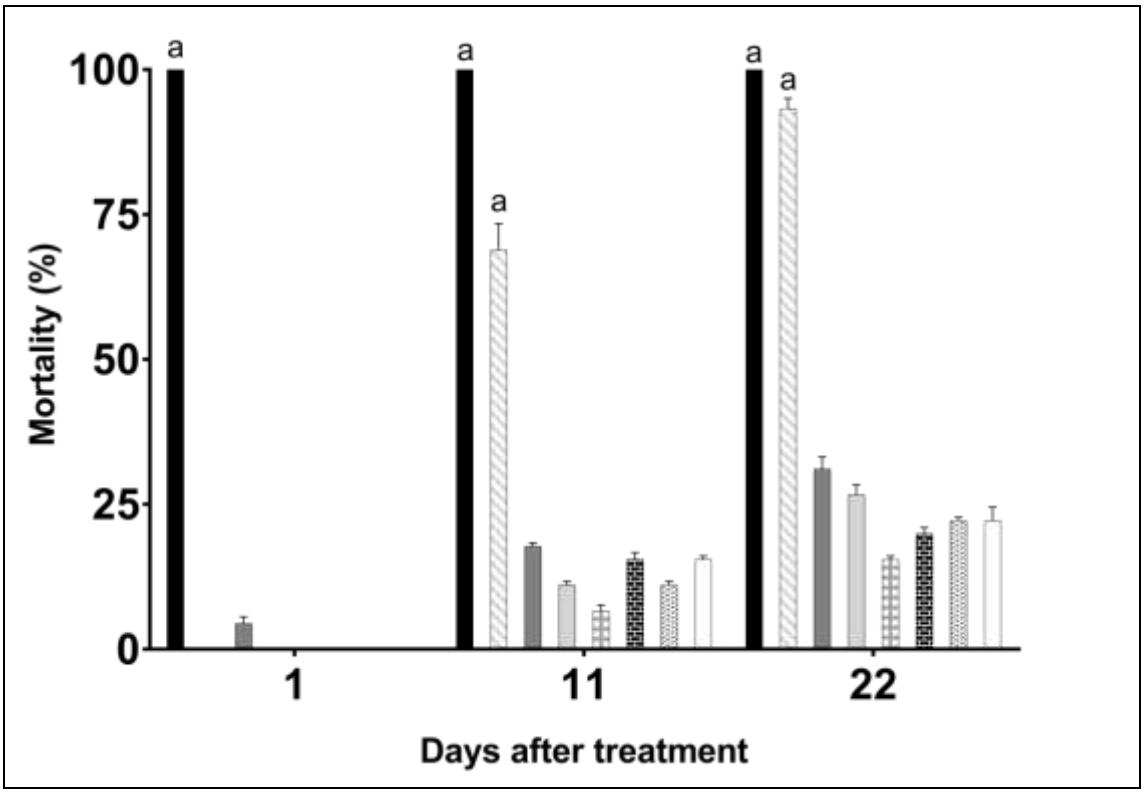
Figure 2. Percentage of mortality after topical treatment **(**1 µL) of *Oncopeltus fasciatus* fourth-instar nymphs with pure essential oil (EO) and their different concentrations obtained from the leaves of *Pilocarpus spicatus.* ☐ Untreated control group; ▩ Solvent control group; ■ 1.2 mg (Pure EO); ▨ 0.6 mg/mL; ■ 0.3 mg/mL; ▭ 0.15 mg/mL; ▦ 0.075 mg/mL; ▬ 0.0375 mg/mL. Values are means ± SD of at least three experiments with 30 insects each. Superscript letters show statistical differences between treated and control insects: a - p <0.0001 (Tukey's test).



Source: Authors.

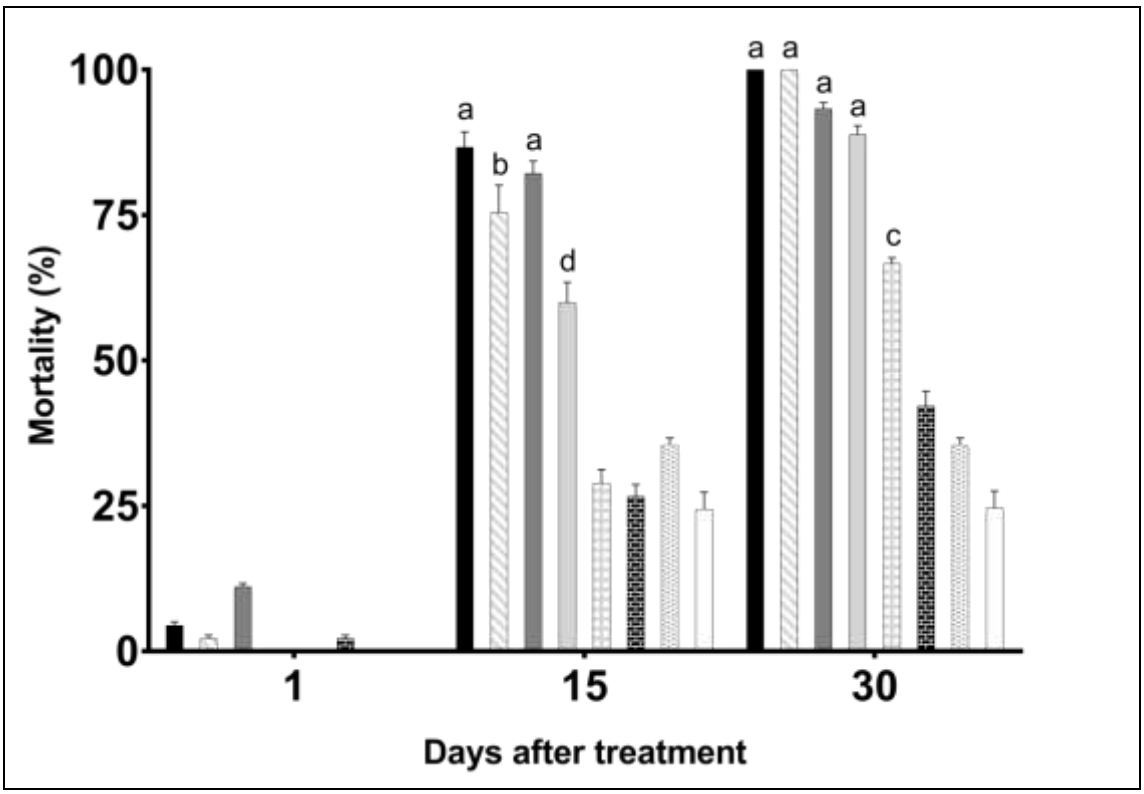
Figure 3 shows that for continuous treatment, *D. peruvianus* presented 22.20±0.58% (p >0.05) of mortality in solvent control group and 22.20±2.31% in untreated control group at 22 days after application. At the same period, 20.0±1.00% (p >0.05), 15.53±0.58% (p >0.05), 26.67±1.73% (p >0.05), 31.13±2.08% (p >0.05) and 93.33±1.73% (p <0.0001) of mortality were recorded to insects treated with 13 µg/cm^2, 26 µg/cm^2, 52 µg/cm^2, 104 µg/cm^2 and 207 µg/cm^2 of EO, respectively. None of the insects survived 24 h after the treatment with 415 µg/cm^2 (pure EO) (p <0.0001).

Figure 3. Percentage of mortality after continuous treatment of *Dysdercus peruvianus* fourth-instar nymphs with pure essential oil (EO) and their different concentrations obtained from the leaves of *Pilocarpus spicatus.* ☐ Untreated control group; ▩ Solvent control group; ■ 415 µg/cm^2 (Pure EO), ▨ 207 µg/cm^2; ■ 104 µg/cm^2; ▭ 52 µg/cm^2; ▦ 26 µg/cm^2; ▬ 13 µg/cm^2. Values are means ± SD of at least three experiments with 15 insects each. Superscript letters show statistical differences between treated and control insects: a - p <0.0001 (Tukey's test).



Source: Authors.

Figure 4. Percentage of mortality after continuous treatment of *Oncopeltus fasciatus* fourth-instar nymphs with pure essential oil (EO) and their different concentrations obtained from the leaves of *Pilocarpus spicatus.* ☐ Untreated control group; ▩ Solvent control group; ■ 415 µg/cm^2 (Pure EO); ▨ 207 µg/cm^2; ■ 104 µg/cm^2; ▭ 52 µg/cm^2; ▦ 26 µg/cm^2; ▬ 13 µg/cm^2. Values are means ± SD of at least three experiments with 15 insects each. Superscript letters show statistical differences between treated and control insects: a - p <0.0001; b – p <0.001; c – p <0.01; d – p <0.05 (Tukey's test).



Source: Authors.

As shown in Figure 4, *O. fasciatus* displayed 35.53±1.15% (p >0.05) of mortality in solvent control insects and 24.67±2.89 in untreated control group at 30 days of treatment. For EO concentrations of 104 µg/cm^2, 52 µg/cm^2, 26 µg/cm^2 and 13 µg/cm^2, *O. fasciatus*, at the same period of observation, showed 93.33±1.00% (p <0.0001), 88.87±1.53% (p <0.0001), 66.67±1.00% (p <0.01) and 42.20±2.52% (p >0.05) of mortality, respectively. None of the insects survived 24h after the treatment with 415 µg/cm^2 EO (p <0.0001), and neither after treatment with 207 µg EO/cm^2 (p <0.0001) at 30 days.

The toxicity of *P. spicatus* EO was also expressed as the concentration at which 50% and 90% of insects were killed in a specified time (LD50 and LD90, respectively). Table 2 shows that LD50 values to *D. peruvianus* were 90 µg/mL for topical treatment and 110.88

µg/cm^2 for continuous treatment at 22 days of treatment. LD90 values were 310 µg/mL in topical treatment and 221.08 µg/cm^2 in continuous treatment. For *O. fasciatus,* LD50 values were 9 µg/mL for topical treatment and 10.90 µg/cm^2 for continuous treatment at 30 days of treatment, and LD90 values recorded at the same time were 90 µg/mL for topical treatment and 76.92 µg/cm^2 for continuous treatment. It is worth to note that both topical and continuous treatments induced higher lethality to *O. fasciatus* in comparison to *D. peruvianus.* For both treatments, the highest oil concentrations used were able to kill all tested nymphs.

Thus, the effects of either topical or continuous treatment of *P. spicatus* EO on both species were often displayed in a dose response manner.

Table 2. Determination of median lethal dose (LD50) and lethal dose 90% (LD90) after *Pilocarpus spicatus* essential oil (EO) treatment of *Dysdercus peruvianus* and *Oncopeltus fasciatus* fourth-instar nymphs. Insects received different EO dilutions which were applied topically or assayed by continuous treatment. The values were calculated based on the end of experiments for each insect species.

	Dysdercus peruvianus		*Oncopeltus fasciatus*	
	Topical treatment	Continuous treatment	Topical treatment	Continuous treatment
Period	22 days	22 days	30 days	30 days
Estimated LD50	90 µg/mL	110.88 µg/cm^2	9 µg/mL	10.90 µg/cm^2
Lower 95.0%	60 µg/mL	94.27 µg/cm^2	- 28 µg/mL	- 11.19 µg/cm^2
Upper 95.0%	110 µg/mL	131.51 µg/cm^2	28 µg/mL	22.94 µg/cm^2
Estimated LD90	310 µg/mL	221.08 µg/cm^2	90 µg/mL	76.92 µg/cm^2
Lower 95.0%	270 µg/mL	189.46 µg/cm^2	80 µg/mL	61.69 µg/cm^2
Upper 95.0%	390 µg/mL	271.23 µg/cm^2	120 µg/mL	108.89 µg/cm^2

Source: Authors.

Table 3 represents the disruption of development in *D. peruvianus* submitted to topical and continuous treatment with *P. spicatus* EO and its dilutions. Firstly, no deformities or developmental delay were observed in both control groups (untreated and solvent only) submitted to either topical or continuous treatment or in insects topically treated with 1.2 mg EO. Insects topical treated with 0.6 mg EO/mL displayed 1.11±6.08% ($p < 0.05$) of deformed adults and 1.11±6.08% ($p < 0.05$) of supernumerary nymphs (sixth-instar nymphs) while 0.3 mg EO/mL induced 0.37±7.64% ($p > 0.05$) of deformed adults. For 0.15 mg EO/mL concentration, 0.37±7.64% ($p > 0.05$) of deformities in fifth-instar nymphs and 0.74±7.02% ($p > 0.05$) of deformed adults were recorded. Topical application of 0.075 mg EO/mL induced 1.11±6.08% ($p < 0.05$) of deformed adults while 0.0375 mg EO/mL displayed 0.37±7.64% ($p > 0.05$) of deformed adults and 0.37±7.64% ($p > 0.05$) of permanent fourth-instar insects. For continuous treatment 2.22±8.14% ($p < 0.05$) of deformities in fifth-instar and 0.74±5.67% ($p > 0.05$) of deformed adults were observed after treatment with 207 µg EO/cm^2. The 104 µg EO/cm^2 dose induced 0.74±5.67% ($p > 0.05$) of deformity in fifth-instar nymphs.

As demonstrated in Table 4, no deformities or developmental delay are observed in *O. fasciatus* in control groups (untreated and solvent only) submitted to either topical or continuous treatment. After topical treatment of *O. fasciatus* with 0.6 mg EO/mL, 0.37±7.64% ($p > 0.05$) of permanent fourth-instar nymphs and 0.37±7.64% ($p > 0.05$) of permanent fifth-instar nymphs were recorded. Treatment with 0.3 mg EO/mL induced 0.37±7.64% ($p > 0.05$) of deformity in fifth-instar nymphs, 0.37±7.64% ($p > 0.05$) of permanent fifth-instar nymphs, 0.37±7.64% ($p > 0.05$) of supernumerary nymphs and 0.37±7.64% ($p > 0.05$) of deformed adults. When we used 0.15 mg EO/mL, 0.74±7.02% ($p > 0.05$) of deformed adults were observed. 0.075 mg EO/mL dose induced 0.37±7.64% ($p > 0.05$) of deformities in fifth-instar nymphs and 1.11±6.08% ($p < 0.05$) of deformed adults. Finally, topical treatment with 0.0375 mg EO/mL displayed 0.37±7.64% ($p > 0.05$) of permanent fourth-instar nymphs, 2.59±4.53% ($p < 0.05$) of deformed fifth-instar nymphs, 0.74±7.02% ($p > 0.05$) of permanent fifth-instar nymphs and 0.37±7.64% ($p > 0.05$) of deformed adults.

For continuous treatment, 4.44±3.51% ($p < 0.05$) of deformity in fifth-instar nymphs were observed after treatment with 415 µg EO/cm^2. 207 µg/cm^2 concentration induced 2.96±7.23% ($p < 0.05$) of deformities in fifth-instar nymphs and 0.74±5.67% ($p > 0.05$) of deformed adults. Treatments of 104 µg/cm^2 resulted in 2.96±7.23% ($p < 0.05$) of deformities in fifth-instar nymphs and 0.74±5.67% ($p > 0.05$) of deformed adults. After 52

µg/cm^2 concentration test, 4.44±3.51% ($p < 0.05$) of deformed adults were recorded. 26 µg/cm^2 concentration expressed 1.48±7.37% ($p < 0.05$) of deformity in fifth-instar insects and 1.48±7.37% ($p < 0.05$) of deformed adults; and 13 µg/cm^2 displayed 1.48±7.37% ($p < 0.05$) of deformity in fifth-instar insects and 2.22±6.43% ($p < 0.05$) of deformed adults. There were no permanent-instar insects and supernumerary nymphs evidenced in continuous treatment, neither deformations in controls of both treatments.

Table 3. Disruption of development in *Dysdercus peruvianus* topical and continuous treatment with *Pilocarpus spicatus* essential oil (EO) and its dilutions. Values are means ± SD of at least three experiments of triplicates with 30 (topical treatment) and 15 (continuous treatment) insects each. Letters show statistical differences between treated and control insects: a - $p < 0.05$ (Tukey's test).

Treatment	EO concentration	Permanent fourth-instar (% ± SD)	Deformed fifth-instar (% ± SD)	Permanent fifth-instar (% ± SD)	Supernumerary nymphs (sixth-instar) (% ± SD)	Deformed adults (% ± SD)
Topical	0.0375 mg/mL	0.37±7.64	0	0	0	0.37±7.64
	0.075 mg/mL	0	0	0	0	1.11±6.08a
	0.15 mg/mL	0	0.37±7.64	0	0	0.74±7.02
	0.3 mg/mL	0	0	0	0	0.37±7.64
	0.6 mg/mL	0	0	0	1.11±6.08a	1.11±6.08a
	1.2 mg	0	0	0	0	0
	Solvent control group	0	0	0	0	0
	Untreated control group	0	0	0	0	0
Continuous	13 µg/cm^2	0	0	0	0	0
	26 µg/cm^2	0	0	0	0	0
	52 µg/cm^2	0	0	0	0	0
	104 µg/cm^2	0	0.74±5.67	0	0	0
	207 µg/cm^2	0	2.22±8.14a	0	0	0.74±5.67
	415 µg/cm^2	0	0	0	0	0
	Solvent control group	0	0	0	0	0
	Untreated control group	0	0	0	0	0

Source: Authors.

Table 4. Disruption of development in *Oncopeltus fasciatus* topical and continuous treatment with *Pilocarpus spicatus* essential oil (EO) and its dilutions. Values are means ± SD of at least three experiments of triplicates with 30 (topical treatment) and 15 (continuous treatment) insects each. Letters show statistical differences between treated and control insects: a - $p < 0.05$ (Tukey's test).

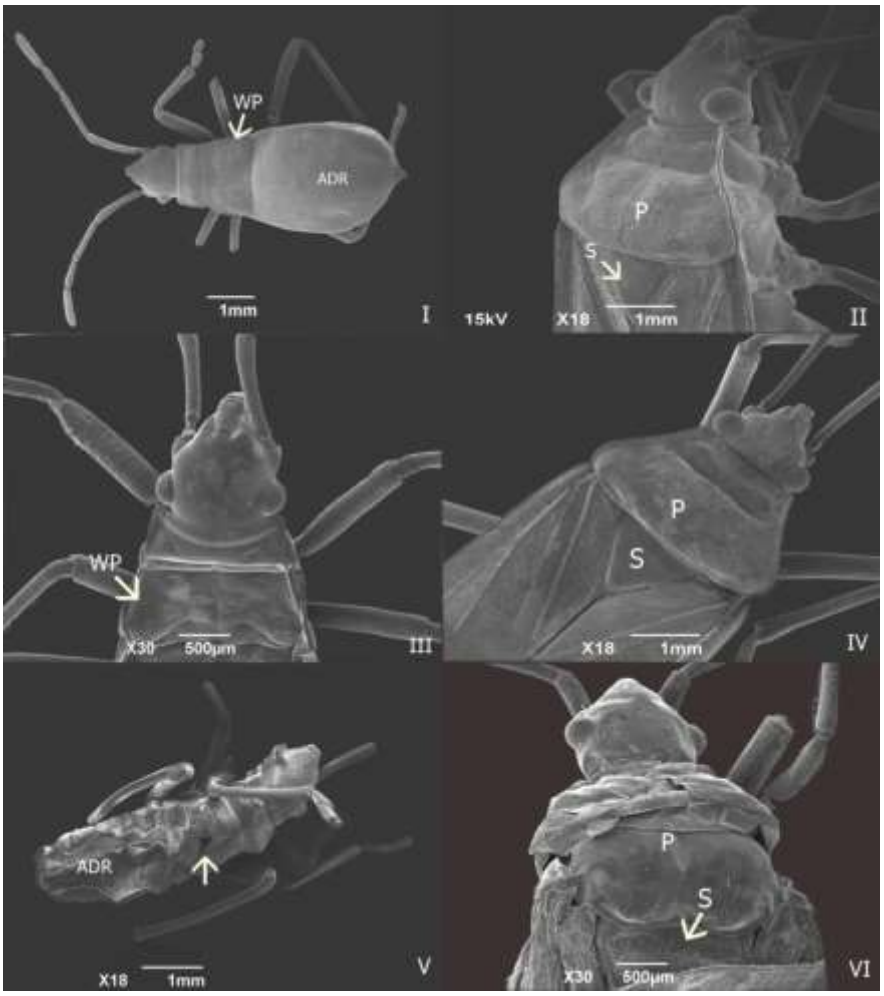
Treatment	EO concentration	Permanent fourth-instar (% ± SD)	Deformed fifth-instar (% ± SD)	Permanent fifth-instar (% ± SD)	Supernumerary nymphs (sixth-instar) (% ± SD)	Deformed adults (% ± SD)
Topical	0.0375 mg/mL	0.37±7.64	2.59±4.53a	0.74±7.02	0	0.37±7.64
	0.075 mg/mL	0	0.37±7.64	0	0	1.11±6.08a
	0.15 mg/mL	0	0	0	0	0.74±7.02
	0.3 mg/mL	0	0.37±7.64	0.37±7.64	0.37±7.64	0.37±7.64
	0.6 mg/mL	0.37±7.64	0	0.37±7.64	0	0
	1.2 mg	0	0	0	0	0
	Solvent control group	0	0	0	0	0
	Untreated control group	0	0	0	0	0
Continuous	13 µg/cm^2	0	1.48±7.37a	0	0	2.22±6.43a
	26 µg/cm^2	0	1.48±7.37a	0	0	1.48±7.37a
	52 µg/cm^2	0	0	0	0	4.44±3.51a
	104 µg/cm^2	0	2.96±7.23a	0	0	0.74±5.67
	207 µg/cm^2	0	2.96±7.23a	0	0	0.74±5.67
	415 µg/cm^2	0	4.44±3.51a	0	0	0
	Solvent control group	0	0	0	0	0
	Untreated control group	0	0	0	0	0

Source: Authors.

Figures 5 and 6 show scanning electron micrographs of both control and treated *D. peruvianus* and *O. fasciatus.* Regions such as the wing pads (WP), abdominal dorsal region (ADR), pronotum (P) and scutellum (S) were focused. The normal morphology observed in nymphs of fourth and fifth-instar and adult insects of untreated control group are similar to those of the solvent control group. However, malformations such as depressions in dorsal region, absence of wings pads, atrophy of wings, abdomen and head, curvature of

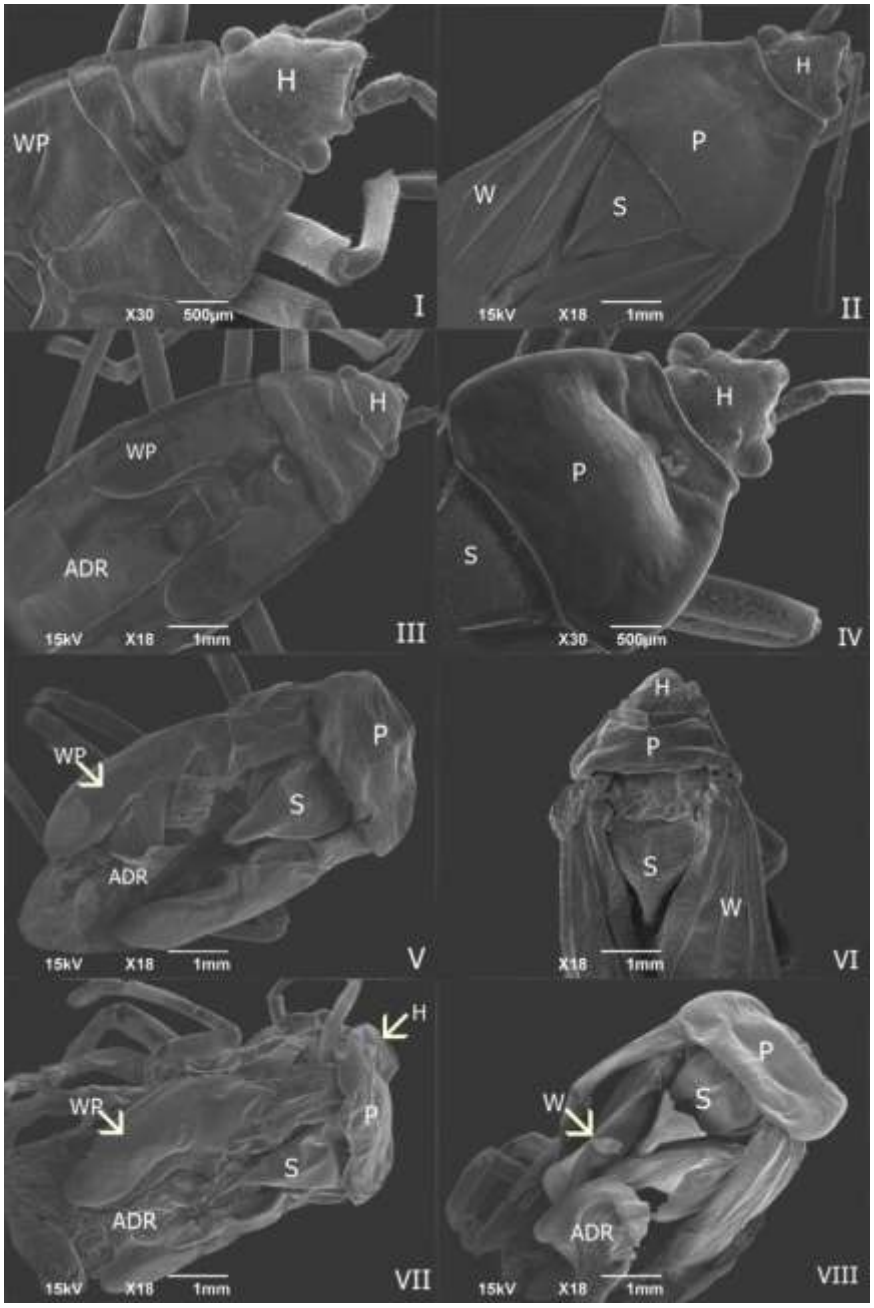
pronotum and incomplete formation of scutellum were observed after different treatments with *P. spicatus*-EO concentrations.

Figure 5. Electron micrography of *Dysdercus peruvianus* insects submitted to treatments with *Pilocarpus spicatus* essential oil. Fourth-instar and adult of untreated control group – I and II (x18); fourth-instar and adult of solvent control group – III (x30) and IV (x18); permanent fourth-instar nymph after topical treatment with 0.0375 mg/mL of *Pilocarpus spicatus* essential oil, showing a depression located in the dorsal region – V (x18); adult obtained from topical treatment with 0.0375 mg/mL essential oil – VI (x30). Wing pads (WP), abdominal dorsal region (ADR), pronotum (P) and scutellum (S).



Source: Authors.

Figure 6. Electron micrography of *Oncopeltus fasciatus* insects submitted to treatments with *Pilocarpus spicatus* essential oil. Fifth-instar and adult of untreated control group – I (x30) and II (x18); fifth-instar and adult of solvent control group (acetone) – III (x18) and IV (x30); fifth-instar obtained from topical treatment with 0.0375 mg/mL of *Pilocarpus spicatus* essential oil – V (x18); adult obtained from continuous treatment with 13 µg/cm^2 essential oil – VI (x18); fifth-instar obtained from topical treatment with 0.3 mg/mL essential oil – VII (x18); adult obtained from continuous treatment with 52 µg/cm^2 essential oil – VIII (x18).Wing pads (WP), abdominal dorsal region (ADR), pronotum (P), scutellum (S), head (H), wings (W).



Source: Authors.

4. Discussion

In a previous work, using the hematophagous kissing bug *Rhodnius prolixus* as a model, our group observed a high insecticidal activity of *P. spicatus* EO when nymphs were treated topically or orally, with the addition of EO in the blood meal (Mello, et al., 2007). Herein, using two phytophagous hemipteran as models, our results showed that a single topical treatment of pure or diluted *P. spicatus* EO not only induced high lethality with much lower doses but also significantly disrupted the development of both *O. fasciatus* and *D. peruvianus* and resulted either in permanent over-aged nymphs or supernumerary nymphs and often body deformities. Such over-aged or supernumerary nymphs of hemimetabolous insects with greatly extended instar stages can survive for several weeks but are unable to undergo metamorphosis and reproduce (Tietbohl, et al., 2020). These effects are usually induced by compounds that interfere in insect development by a neuroendocrine route, inhibiting the synthesis of the molting hormone, ecdysone (Dorn, et al., 1986; Feder, et al., 2019) or by directly disrupting chitin synthesis (Merzendorfer, 2006). Similar data were also reported in other studies. Fernandes et al. (2013) tested extracts and triterpenes from *Manilkara subsericea* (Mart.) Dubard (Sapotaceae) on *D. peruvianus* and *O. fasciatus* and observed death during ecdysis and adults with deformed wings and wrinkled dorsal cuticle. Saxena & Srivastava (1972) tested the extracts of *Iris ensembles* Thamb. (Iridaceae) on *Dysdercus koenigii* (Fabricius, 1775) and observed adults with wing deformation as well as supernumerary nymphs. Also, using the *D. koenigii* model, Khan & Qamar (2011) performed tests with a chitin synthesis inhibitor substance and observed incomplete molting characterized by a cuticle that was docked to the last segments of the abdomen or the whole body. Deformed nymphs and wing abnormalities were also observed. Bai & Koshy (2004) evaluated the activity of *Thevetia neriifolia* Juss. (Apocynaceae) extracts on *Dysdercus cingulatus* (Fabricius, 1755), and showed deformation in insect molting and mortality. Similar deformities were also observed in *R. prolixus* after oral administration of the juvenil hormone (JH) analogue Precocene II (Azambuja, et al., 1981), pointing out the morphogenetic effects of JH and analogues such as adult insects with abdominal cuticle with larval tergites and sternites, insects with slightly flattened torax, adults with wings reduced to half normal size and normally crumpled, nymphs with slightly crumpled or curved wing pads, and supernumerary nymphs (Wigglesworth, 1969).

In this way, the chemical analysis of *P. spicatus* EO showed sabinene and sylvestrene as the major components, which differs from previous reports of Oliveira et al. (2010) and Santos et al. (1997), who listed limonene as the major component in the EO. Moreover, limonene was not detected in this investigation. Variations in EO composition are often induced by seasonality and soil composition and plants can exhibit remarkable fluctuation in EO contents with the progress of the seasons (Chericoni, et al., 2006; Zoghbi, et al., 2014; Chaubey, 2019). Sylvestrene was recognized as the major component of *Daucus glaber* (Forssk.) Thell. (Apiaceae) (Mansour, et al., 2004). Sabinene is known as one of the major constituent of EO obtained from *Zornia diphylla* (L.) Pers (Fabaceae) (Arunkumar, et al., 2014), *Zanthoxylum monophyllum* (Lam.) P. Wilsom (Rutaceae) (Prieto, et al., 2010), *Hyptis suaveolens* L. (Lamiaceae) (Benelli, et al., 2012), *Elettaria cardamomum* (L.) (Zingiberaceae) (Chegini & Abbasipour, 2017), *Bacopa caroliniana* (Walter) B.L.Rob (Plantaginaceae) (Liu, et al., 2019), *Psidium guajava* (L.) (Myrtaceae) (El-Sabrout, et al., 2019) and *Dracocephalum integrifolium* Bunge (Lamiaceae) (Zhou, et al., 2019); and has been reported to its antimicrobial properties (Arunkumar, et al., 2014), fumigant and contact toxicity (Wang, et al., 2011; Wang, et al., 2015, 2019) and acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) inhibitory activity (Menichini, et al., 2009). In our study, we observed that the insects treated topically with the pure EO showed temporary paralysis just after topical oil application (data not shown). The same results were reported in *R. prolixus* by Mello et al. (2007) which point out that both molt inhibition and transitory paralysis triggered by topical and feeding treatments were related not only to toxicity itself but, probably also due interference of *Pilocarpus* compounds on the triatomine neuroendocrine system. In fact, ecdysis is under neurological control (Wigglesworth, 1934, 1972) and insect movement (Lazzari, 1992; Takano-Lee & Edman, 2001) can be affected by AChE activity (López & Pascual-Villalobos, 2010). Thus, probably the interaction of secondary metabolites present in EO with insect physiological and hormonal processes may be responsible for the changes in metamorphosis and survival, possibly by both AChE and BuChE inhibitory activity and/or alteration of the JH and Prothoracicotropic Hormone (PTTH) levels, as previously observed for other insects (Menichini, et al., 2009; Tiebolth, et al., 2020). In this way, the compounds present in the essential oil might act as insect growth regulators (IGR) since substances with IGR activity, such as azadirachtin, JH analogs and inhibitors of chitin synthesis have been known to disrupt the development of both *O. fasciatus and Dysdercus*

genus (Saxena & Srivastava, 1972; Redfern, et al., 1982; Dorn, 1986; Mordue & Nisbet, 2000; Bai & Koshy, 2004; Khan & Qamar, 2011, 2012; George, et al., 2014).

The efficacy of an EO is direct related to the amounts of chemical constituents and synergism or antagonism among them (Hummelbrunner & Isman, 2001; Savelev, et al., 2003) and the components of *P. spicatus* EO are now under investigation by our group to analyze their potential to disrupt the insect neuroendocrine system since very low doses of the EO can disrupt the insect development. In fact, EOs present some advantages in relation to synthetic substances with just one active principle since the complex mixture contains many components which show different mechanisms of action on the insect development (Tripathi, et al., 2009; Pohlit, et al., 2011). Also, the high toxicity demonstrated in the experiments can be explained by several factors, including the entry of toxins by mechanisms of inhalation, ingestion, and skin absorption (Regnault-Roger, 1997) which should be better determined by additional investigation.

5. Conclusion

In conclusion, this is the first time to our knowledge that the insecticidal activity against the phytophagous Hemiptera *O. fasciatus* and *D. peruvianus* has been reported for the plant species *P.* spicatus. The present results on the various effects of the EO from leaves of *P. spicatus* on the development of *O. fasciatus* and *D. peruvianus* indicate that this oil is a promising candidate to green pest management of crop insects.

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Percentage of contribution of each author in the manuscript

Raul Apolinário – 18,5%

Jeane Nogueira – 18,5%

Meiriellem G. da Silveira Costa – 2%

Jacenir Santos-Mallet – 5%

Marcelo Guerra Santos – 10%

Patrícia Azambuja – 5%

Cícero Brasileiro Mello – 5%

Marcelo Salabert Gonzalez – 12%

Leandro Rocha – 12%

Maria Denise Feder – 12%