Revista Ciência Agronômica, v. 49, n. 3, p. 484-490, jul-set, 2018
Centro de Ciências Agrárias - Universidade Federal do Ceará, Fortaleza, CE
www.ccarevista.ufc.br

Scientific Article
ISSN 1806-6690

Insecticidal action of *Drimys brasiliensis* Miers on black citrus aphid[1]

Ação inseticida de *Drimys brasiliensis* Miers sobre o pulgão-preto dos citros

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ABSTRACT - The aim of this study was to evaluate the insecticidal effects of a hexane fraction and polygodial compound obtained from *Drimys brasiliensis* roots on the mortality of adults and nymphs of *Toxoptera citricida* (black citrus aphid). For the hexane fraction, solutions of 0.12, 0.25, 0.50, 1.0, and 2.0 mg mL^{-1} were prepared, and for the compound, solutions of 62.5, 125, 250, and 500 µg mL^{-1} were prepared. A control with water and dimethylsulfoxide (5 µg mL^{-1}) was also included. Two hexane-fraction-solution application methods were evaluated: spraying directly onto the insect, and submerging a young *Citrus limonia* shoots in the solution, which served as a refuge for the aphids. Nymph and adult mortality were assessed at 24 and 48 h after solution application. The hexane fraction caused high mortality amongst the *T. citricida* nymphs and adults. The lowest LC_{50} value (0.301 mg mL) was recorded in the nymphs when the solutions were applied directly onto the insects. The polygodial compound that was isolated from the hexane fraction significantly increased the mortality of adults and nymphs, with LC_{50} values of 293.8 and 176.77 µg mL^{-1}, respectively. The insecticidal activity observed may have been caused by this compound.

Key words: Casca d'anta. Botanical insecticide. Insect mortality. Polygodial compound.

RESUMO - O objetivo deste estudo foi avaliar a atividade inseticida da fração hexânica e do composto poligodial obtidos de raízes de *Drimys brasiliensis* sobre a mortalidade de adultos e ninfas de um dia de Toxoptera citricida (pulgão-preto dos citros). Para a fração hexânica foram preparadas soluções nas concentrações de 0,12; 0,25; 0,50; 1,0 e 2,0 mg mL^{-1} e, para o composto poligodial, concentrações de 62,5; 125; 250 e 500 µg mL^{-1}. Água e dimetilsulfóxido (5 µg mL^{-1}) foram utilizados como controle. Para a fração hexânica foram testadas duas formas de aplicação: pulverizações sobre os insetos e imersão do ramo caulinar jovem de *Citrus limonia* na solução, o qual serviu como hospedeiro para os pulgões. A avaliação da mortalidade dos adultos e de ninfas foi realizada após 24 e 48 horas da aplicação das soluções. A fração hexânica de raízes ocasionou elevada mortalidade de ninfas e adultos. O menor valor de CL_{50} (0,301 mg mL^{-1}) foi registrado para as ninfas quando as soluções foram aplicadas sobre o inseto. O composto poligodial, isolado da fração hexânica, e aplicado sobre o inseto, aumentou significativamente a mortalidade de adultos e ninfas, com valores de CL_{50} de 293,8 e 176,77 µg mL^{-1}, respectivamente. A ação inseticida pode ser devida à presença deste composto.

Palavras-chaves: Casca-de-anta. Inseticida botânico. Mortalidade de inseto. Composto poligodial.

DOI: 10.5935/1806-6690.20180055
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Received for publication on 12/12/2014; approved on 20/07/2017
[1]Parte da Tese de Doutorado do primeiro autor apresentada ao Programa de Pós-Graduação em Ecologia e Recursos Naturais, Universidade Federal de São Carlos, São Carlos-SP, Brasil
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INTRODUCTION

Brazil is a major producer of citrus and the largest producer of oranges, which are almost all exported for their juice (FRANCO, 2016; SANTOS *et al*., 2013). Therefore, citriculture is one of the main agricultural activities of the country (FRANCO, 2016; NEVES *et al*., 2010). Among the insects that occur in citrus, aphids (Hemiptera: Aphididae) are important because they are vectors of phytopathogenic viruses (TOLEDO; BARBOSA; YAMAMOTO, 2006). *Toxoptera citricida* Kirkaldy (Hemiptera: Aphididae) (black citrus aphid) is one of the main aphids that colonizes citrus plants (BALFOUR; KHAN, 2012). It indirectly damages crops because it is an efficient vector of Citrus tristeza virus (ZANUTTO *et al*., 2013).

Over the past few decades, the use of synthetic pesticides has been the most efficient mechanism for controlling insect pests. However, the continued use of these products can cause problems such as the elimination of beneficial insects, pest population explosions, and in particular, a reduction in insecticidal efficacy due to the natural selection of insect strains that are resistant to these chemical compounds. Environmental contamination is also a problem (MARANGONI; MOURA; GARCIA, 2012). Currently, natural products derived from plants are considered one of the most promising eco-friendly and biorational alternatives to synthetic pesticides, with new modes of action for the management of insect pests (ISMAN, 2006; RATTAN, 2010). Secondary metabolites synthesized by plants have insecticidal, deterrent, or insect repellent properties, and include terpenes, flavonoids, alkaloids, phenols, and other related compounds. These compounds have the advantages of selectivity and are not toxic to the environment (DANG *et al*., 2010; ISMAN, 2006).

Botanical insecticides are derived from the plant material itself, which is normally ground to powder, or its derivatives, which are obtained by aqueous or organic solvent extraction (WIESBROOK, 2004). The extracts obtained using organic solvents contain a complex mixture of active compounds. If a low lethal concentration is detected, the extract can be fractionated in order to isolate the chemical constituent responsible for the effect (SHAALAN *et al*., 2005). The deleterious effects of these natural products can be manifested in a variety of ways: repellency, feeding inhibition, changes in the hormonal system, and the blocking of neurotransmission, which can cause problems with development and result in deformation, infertility, and high mortality in the various insect stages (ROEL, 2001).

Drimys brasiliensis Miers (Winteraceae), or casca d' anta as it is popularly known, is found in the Atlantic forest and gallery forests of the Brazilian Cerrado (EHRENDORFER; SILBERBAUER-GOTTSBERGER; GOTTSBERGER, 1979). Species belonging to the genus *Drimys* are known to contain sesquiterpene compounds of the drimane group, which exhibit a wide variety of biological activities, including high insecticidal potential (JANSEN; GROOT, 2004). Examples of drimanes isolated from *D. brasiliensis* include the polygodial compound 1-β-(p-methoxycinnamoyl)-polygodial and drimanial (MALHEIROS *et al*., 2005). The polygodial compound has bactericidal (SILVEIRA *et al*., 2012), leishmanicidal (CORRÊA *et al*., 2011), and antifungal (MALHEIROS *et al*., 2005) properties.

Few data are available regarding the use of *D. brasiliensis* in controlling insect pests. Therefore, the objective of this study was to evaluate the insecticidal effects of the hexane fraction and polygodial compound isolated from *D. brasiliensis* roots on the mortality of *T. citricida* adults and nymphs.

MATERIAL AND METHODS

D. brasiliensis subsp. *brasiliensis* roots were collected from plants in the Cerrado (tropical savanna) sensu stricto reserve area of the Federal University of São Carlos (UFSCar), São Paulo (21°58′-22°00′ S and 47°51′-47°52′ W) in August 2010. Whole roots were dried in a forced-air-circulation oven at 40 °C for 120 h and ground in an industrial mill, and the resulting powder was used in the experiments. Initially, 100 g of the root powder was subjected to exhaustive extractions with dichloromethane (DCM)/methanol (MeOH) (1:1) (5 × 400 mL) for 24 h at the ambient laboratory temperature. The resulting extract was vacuum-filtered using a porcelain filter lined with filter paper and attached to a Kitasato flask, before being pooled and concentrated under low pressure at 37 °C on a rotatory evaporator. The concentrated crude extract was suspended in 95% MeOH (400 mL) and partitioned with n-hexane (Hex 3 × 400 mL), resulting in MeOH and n-hexane fractions. Finally, the hexane fraction was concentrated and used to prepare the concentrations to be tested in the bioassay. The hexane fraction of *D. brasiliensis* was chosen due to its high biological activity that was observed in preliminary tests.

To purify the polygodial compound, a hexane fraction sample (1 g) was separated by chromatography using a prepacked silica gel column (10 g, Strata™ Phenomenex®), and eluted with a gradient of hexane/ acetone, acetone, acetone/MeOH and MeOH. After analysis based on thin-layer chromatography, nine sub-fractions (A-I) were obtained. Fraction D (hexane/acetone, 9:1 v/v, 160 mg) was selected for purification by reverse-phase high performance liquid chromatography/ultraviolet

light using an analytical column, and seven samples (D1-D7) were obtained. Following ^{1}H and ^{13}C nuclear magnetic resonance analysis and comparison with the literature, sample D3 (86.4 mg) was identified as a polygodial compound (KIOY; GRAY; WATERMAN, 1990), and its biological activity was investigated in bioassays with the insects.

For the preparation of the solutions, the hexane fraction and the polygodial compound were separately pre-solubilized in dimethylsulfoxide (DMSO, 5 µL mL^{-1}) diluted in Milli-Q® water. For the hexane fraction, solutions were prepared in concentrations of 0.12, 0.25, 0.50, 1.0, and 2.0 mg mL^{-1}, and for the isolated compound, concentrations of 62.5, 125, 250, and 500 µg mL^{-1} were used. A control was also included that contained only water and DMSO (5 µg mL^{-1}). Constant concentrations of 5 µL mL^{-1} of DMSO were maintained at each concentration assessed. Prior to performing the bioassay, the effect of DMSO diluted in Milli-Q® water (5 µg mL^{-1}) was tested on the *T. citricida* nymphs and adults, and no changes in behavior, development, or mortality were observed.

T. citricida adults were collected from *Citrus limonia* Osbeck plants at the Campus of the Universidade Federal de São Carlos and taken to the Laboratory of Economic Entomology of the Department of Ecology and Evolutionary Biology for identification, before being placed in young shoots of the same host. The young shoots, individualized, were placed in an Eppendorf tube (2 mL) containing water, and the whole assembly was kept in a glass sleeve with one end sealed with voile fabric. Adults and day-old nymphs were used in the bioassays.

The experiment was conducted in the laboratory, with an average temperature of 27.5 °C and a relative humidity of 60 ± 10%. Two methods of applying the hexane fraction solutions were used: spraying directly onto *T. citricida* adults and nymphs, and submerging young *C. limonia* shoots, insect host, in solutions of the fraction. For the polygodial compound, only direct spraying was performed on the nymphs and adults.

Spraying was performed with a spray bottle by applying 2 mL of solution per replicate (five insects). Subsequently, the insects were transferred to the young shoots of the uncontaminated host using an entomological brush and enclosed in the glass sleeve set and voile fabric. For application on the young shoots, a 10-mL solution was added to a Petri dish (15 cm diameter) that formed a residual layer, in which the young shoots were dipped for 20 s before being allowed to dry for 30 min at room temperature. Uncontaminated insects were then placed on the young shoots, which were enclosed in the glass sleeve set and voile fabric.

In the two forms of application of the solutions, separately, the bioassays with the hexane fraction and with the polygonal compound were carried out in a completely randomized experimental design. Each treatment (different concentrations of the hexane fraction and the polygodial compound) included five replicates and each was composed of a glass sleeve set, voile fabric, the young host shoots, and five insects (adults or nymphs). Adult and nymph mortality were recorded at 24 and 48 h after the solutions had been applied. Insects were considered dead if they did not respond to the touch of the entomological brush.

The mean mortality data after 48 h of exposure to the solutions were subjected to an analysis of variance, and the means were compared by the Scott Knott test at a probability level of 5%. Median lethal concentrations (LC_{50}) were estimated using probit analysis with the POLO-PC program.

To evaluate mean mortality as a function of time after spraying (24 and 48 h), a joint data analysis was performed, because the ratio between the largest and smallest mean residual square did not exceed seven (PIMENTEL-GOMES, 1990).

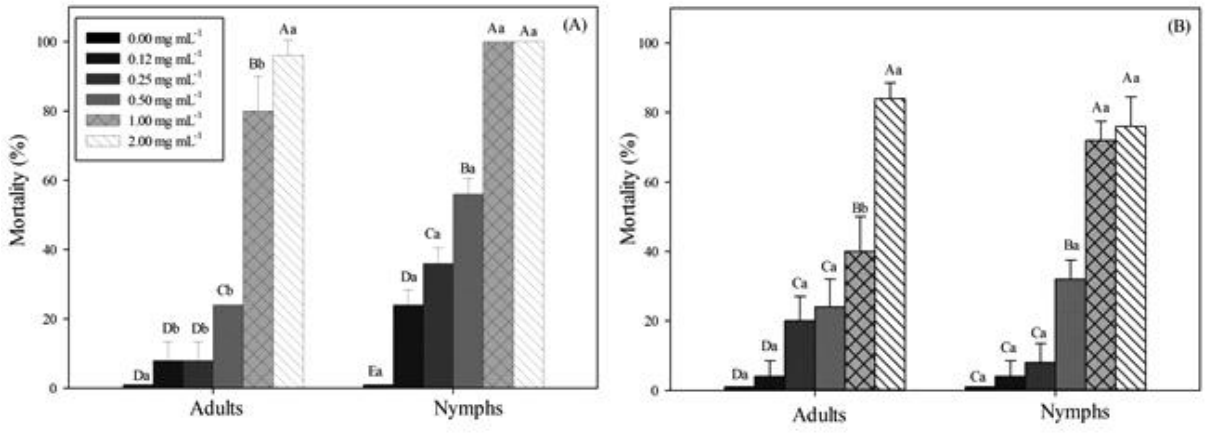
RESULTS AND DISCUSSION

We found that the hexane fraction from a D. brasiliensis root extract exhibited strong insecticidal activity against black citrus aphid. Significant increases were observed in nymph and adult mortality rates that were positively associated with increases in the solution concentration of the hexane fraction, in both forms of application. When the solutions were directly applied to the insects, the maximum mortality rates of adults (96%) and nymphs (100%) were observed at concentrations of 2.0 and 1.0 mg mL^{-1}, respectively (Figure 1A). Similarly, when applied to the young shoots of the host, 86 and 76% mortality of adults and nymphs, respectively, occurred at a concentration of 2.0 mg mL^{-1} (Figure 1B).

When comparing the sensitivity of adults and nymphs to the effects of the hexane fraction applied on to the insects, it was observed that the nymphs were more sensitive to its toxic properties at all concentrations of the fraction, except the highest (2.0 mg mL^{-1}). When the hexane fraction was applied to the host, there was no significant difference in sensitivity between the two stages of aphid development, except at a concentration of 1.0 mg mL^{-1} (Figure 1A and 1B).

The hexane fraction directly applied onto the insects and onto the young shoots of the host resulted in LC_{50} values of 0.620 and 0.932 mg mL^{-1} for adults,

Figure 1 - Mortality of *Toxoptera citricida* adults and nymphs subjected to applications of the hexane fraction from *Drimys brasiliensis* roots directly onto the insects (A) or onto young shoots of the host (B) after 48 h of exposure. The uppercase letters compare concentrations within each stage of development, and the lowercase letters compare concentrations between stages. Bars with the same letter did not significantly differ according to a Scott-Knott test at the 5% probability level



respectively, and of 0.301 and 0.783 mg mL^{-1} for nymphs, respectively (Table 1). The LC_{50} values of the solution that was directly applied onto the insects were lower than those recorded for the solution that was applied to the young shoots of the host. Topical action is one of the main forms of interaction of plant derivatives, such as essential oils (KNAAK; FIUZA, 2010). By direct contact, botanical derivatives can interact with the integument of the insect, as well as affect digestive and neurological enzymes (ISMAN, 2006), resulting in death.

The LC_{50} values also revealed that, for both forms of application, nymphs were more sensitive to the toxic activity of the hexane fraction of *D. brasiliensis* than adults. Tang, Weathersbee and Mayer (2002) reported higher LC_{50} values of a commercial insecticide obtained from neem seeds (Neemix, 4.5% azadirachtin, AZ) for adult *T. citricida* (30.37 ppm) than nymphs (3.9 ppm) after 4 days of exposure to the product.

Regarding effective concentrations of botanical extracts for the control of insect pest species, previous studies have reported LC_{50} values ranging from 0.45 to 20 mg mL^{-1} (CRUZ-ESTRADA *et al*., 2013; GONZAGA *et al*., 2008; MUNGENGE *et al*., 2014). In the present study, the maximum LC_{50} value obtained was 0.93 mg mL^{-1}, so the effective concentrations evaluated in the present study can be considered low, indicating the potential of *D. brasiliensis* as a donor of compounds with insecticidal potential.

By jointly analyzing the mean mortality values of *T. citricida* adults and nymphs as a function of the time elapsed after application of the hexane fraction, we found that nymphs were more sensitive than adults in the first 24 h after exposure, for both forms of application. The nymphs may have been more sensitive than the adults because they possess thin exoskeletons at this stage of development, which may facilitate the penetration of

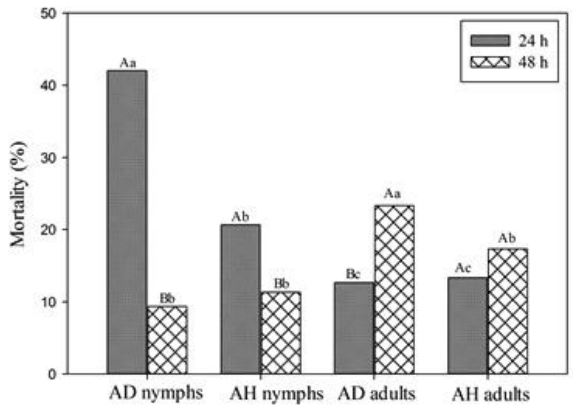
Table 1 - Angular coefficients, lethal concentrations (LC_{50}, in mg mL^{-1}), and confidence intervals of *Toxoptera citricida* adults and nymphs after 48 h of exposure to solutions of the hexane fraction from *Drimys brasiliensis* roots applied directly onto the insect or onto the host

Phase	Angular coefficient (± EE)[a]	LC_{50} (CI)[b]	$X^{2\,(c)}$
Application on insect			
Adults	2.96 ± 0.42	0.620 (0.471-0.836)	3.62
Nymphs	2.68 ± 0.42	0.301 (0.231-0.383)	10.32
Application on host			
Adults	1.98 ± 0.34	0.932 (0.691-1.390)	18.54
Nymphs	2.32 ± 0.36	0.783 (0.602-0.990)	16.26

[a]EE: standard error of the mean. [b]CI: confidence interval at 95% probability of error. [(c)]X^2: chi-square value

insecticides into the cuticle of the insect, as reported by Redoan *et al*. (2010). At the end of the 24 h period, the maximum mortality (42%) was observed in the sprayed nymphs. In contrast, for the adult aphids, mortality was high after 48 h of exposure (Figure 2). The death of the insects within a short period of time demonstrates the immediate effect of the hexanic fraction of *D. brasiliensis*, particularly on *T. citricida* nymphs. This should be taken into account when considering the possible loss of the product by the action of rain after application, as highlighted by Gonzaga *et al*. (2008).

Figure 2 - Mortality (%) of *Toxoptera citricida* nymphs and adults after 24 and 48 h of exposure to solutions of the hexane fraction from *Drimys brasiliensis* roots applied directly onto the insect (AD) or onto young shoots of the host (AH). The uppercase letters compare exposure times within each treatment, and the lowercase letters compare exposure times between treatments. Bars with the same letter did not significantly differ according to a Scott-Knott test at the 5% probability level



The *T. citricida* adults and nymphs were highly sensitive to the effects of the hexane fraction being sprayed directly onto them, although when applied to the host it also caused high mortality rates. Therefore, the results of this study indicate that the hexane fraction from *D. brasiliensis* roots exhibits high insecticidal activity. Insecticidal properties have been reported in other species of the genus *Drimys* (ZAPATA *et al*., 2009, 2010). The significant effect of the hexane fraction on *T. citricida* mortality can be explained by the presence of sesquiterpene compounds of the drimane group. The fractionation of hexane from *D. brasiliensis* stem extracts has allowed the identification of substances of this class of compound, particularly polygodial compounds (CORRÊA *et al*., 2011; LAGO *et al*., 2010; MALHEIROS *et al*., 2005). Drimane compounds that have been isolated from plants are reported to exhibit a great variety of biological activities, particularly against herbivores (JANSEN; GROOT, 2004).

In the present study, fractionation of the hexane fraction from *D. brasiliensis* roots resulted in the isolation of the polygodial compound, which had a strong insecticidal effect on *T. citricida* adults and nymphs. Mortality significantly increased with increasing concentrations of the compound. The maximum mortality rates for nymphs (96%) and adults (76%) were observed at the highest concentration tested (500 µg mL^{-1}). Similar to the hexane fraction results, nymphs were more sensitive to the polygodial compound than adults (Figure 3).

The LC_{50} values using the polygodial compound were 293.8 and 176.77 µg mL^{-1} for adults and nymphs, respectively (Table 2), confirming its insecticidal potential for the control of this aphid. In a previous study, Montenegro *et al*. (2013) reported that the compound caused the mortality of *Drosophila melanogaster* larvae, with an LC_{50} of 60 mg L^{-1}.

The polygodial compound has been reported by several authors to be a potent feeding inhibitor in several species of insect, including *Spodoptera littoralis* (Lepidoptera: Noctuidae) (ZAPATA *et al*., 2009), *Bemisia tabaci* (Hemiptera: Aleyrodidae) (PROTA; BOUWMEESTER; JONGSMA, 2013), and *Nasonovia ribisnigri* (Hemiptera: Aphididae) (ZAPATA *et al*., 2010). Feeding inhibition caused by drimane

Figure 3 - Mortality of *Toxoptera citricida* adults and nymphs subjected to different concentrations of the polygodial compound isolated from *Drimys brasiliensis* roots after 48 h of exposure. The uppercase letters compare concentrations within each stage of development, and the lowercase letters compare concentrations between stages. Bars with the same letter did not significantly differ according to a Scott-Knott test at the 5% probability level

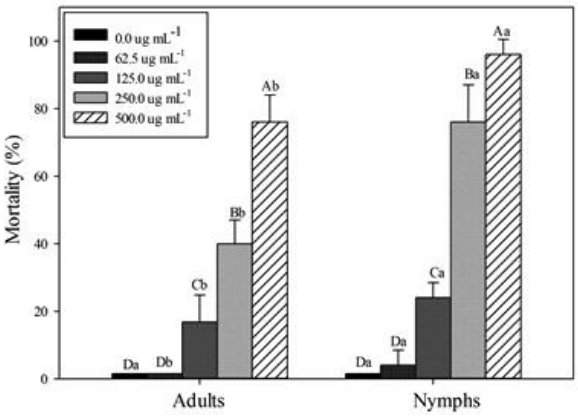


Table 2 - Angular coefficients, lethal concentrations (LC_{50}, in μg mL^{-1}), and confidence intervals of *Toxoptera citricida* adults and nymphs after 48 h of exposure to the polygonal compound isolated from *Drimys brasiliensis* roots by direct application onto the insects

Phase	Angular coefficient (± EE)a	LC_{50} (CI)[b]	$X^{2\,(c)}$
Adults	3.15 ± 0.58	293.86 (234.9-388.9)	10.87
Nymphs	4.07 ± 0.64	176.77 (145.6-214.6)	16.07

[a]EE: standard error of the mean. [b]CI: confidence interval at 95% probability of error. [(c)]X^2: chi-square value

compounds, including polygodial, is related to the effects on chemoreceptors (MORENO-OSORIO *et al*., 2008). Although the polygodial compound acts mainly by inhibiting feeding, some authors have reported that, when ingested, the compound can affect locomotion and digestion, indicating a post-ingestion toxic effect, which results in reduced growth and/or mortality (ZAPATA *et al*., 2009).

The hexane fraction from *D. brasiliensis* roots and the polygodial compound that was isolated from it caused toxicity to *T. citricida*, with high mortality in the adult and nymph stages after 48 h of exposure to the solutions. Our results indicate that the insecticidal effect of the hexane extract from *D. brasiliensis* roots is probably caused by the presence of the polygodial compound. However, even if the toxicity of *D. brasiliensis* to *T. citricida* is due to the presence of this drimane, it is important to note that, from the economic and agroecological point of view, the use of raw or semi-purified plant extracts may be more convenient. Possible synergism between sesquiterpenes and other classes of metabolite cannot be excluded, which would increase their activity (ZAPATA *et al*., 2009). In addition, the use of extracts with a wide variety of metabolites delays agricultural pests developing resistance (ISMAN, 2006; RATTAN, 2010) and is a viable option, particularly for small, rural producers. Considering the results obtained by this study, *D. brasiliensis* should be considered a promising species for the development of botanical insecticides for the sustainable, integrated management of the black citrus aphid.

CONCLUSIONS

1. The hexane fraction of a crude extract from *D. brasiliensis* roots caused high mortality in *T. citricida* nymphs and adults;
2. The polygodial compound isolated from the hexane fraction significantly increased the mortality of *T. citricida* in a dose-dependent concentration.

ACKNOWLEDGEMENTS

We thank CAPES (scholarships), FAPESP (financial support), and Roberto Gomes Souza Berlinck (Chemical Institute of São Carlos-IQSQ/USP) for the guidance, materials, and equipment needed to obtain the hexane fraction and polygodial compound, and Antonio Gilberto Ferreira (Chemistry Department of UFSCar) for the nuclear magnetic resonance spectroscopic analysis.

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