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Dejane Santos Alves[1a+], Pilar Canela Garcia Fernandez[2a], Alejandro Martin Martin[2b], Flor Budia[2c], Geraldo Andrade Carvalho[1b], Ronald Zanetti[1c], Denilson Ferreira de Oliveira[1d]

TOXICITY OF ALKALOID FRACTIONS FROM *Psychotria* spp. (RUBIACEAE) AGAINST *Atta sexdens* FOREL, 1908 (HYMENOPTERA: FORMICIDAE)

ALVES, D. S.; FERNANDEZ, P. C.G.; MARTIN, A. M.; BUDIA, F.; CARVALHO, G. A.; ZANETTI, R.; OLIVEIRA, D. F. Toxicity of alkaloid fractions from *Psychotria* spp. (Rubiaceae) against *Atta sexdens* Forel, 1908 (Hymenoptera: Formicidae). **CERNE**, v. 25, n. 2, p.255-262, 2019.

HIGHLIGHTS

The alkaloid fractions from *Psychotria* spp. were tested against *Atta sexdens*.

The root fractions from *Psychotria hastisepala* presented the highest toxicity against *A. sexdens*.

Branches, stems and leaves fractions of *P. hastisepala* were also toxic to *A. sexdens*.

Psychotria hastisepala have potential to be employed in the *A. sexdens* control.

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ABSTRACT

Leaf-cutting ants are the main pests in forest plantations. The most commonly used chemical control for the ants is toxic bait. However, the active ingredients in these baits have been restricted by forest certification organizations, justifying the search for new active compounds to control these insects. Thus, this work aimed to evaluate the formicidal activity of alkaloid fractions from the roots, stems, branches and leaves of *Psychotria hastisepala* and *Psychotria leiocarpa* (Rubiaceae) against *Atta sexdens* Forel, 1908 (Hymenoptera: Formicidae). The alkaloid fractions were obtained from the crude methanolic extracts of *P. hastisepala* and *P. leiocarpa* by the acid-base extraction method employing liquid-liquid partitions. The fractions, previously solubilized in propanone, were applied topically to the pronotum of worker ants. Bioassays were conducted in a completely randomized design, with six replicates of each treatment and 10 ants per replicate. Insect survival was assessed daily for 21 days. The median lethal time was estimated by Weibull models. The root fractions from *P. hastisepala* presented the highest toxicity, followed by those from the branches, stems and leaves. Among fractions from *P. hastisepala* roots, the most toxic were a fraction that apparently is rich in lipophilic compounds and nonbasic alkaloids and a fraction that is likely rich in quaternary benzophenanthridine alkaloids, protopine alkaloids and tertiary bases. *Psychotria leiocarpa* fractions were not toxic to this insect. *Psychotria hastisepala* is potentially useful for the development of new products for *A. sexdens* control.

+Correspondence:
dejanealves@utfpr.edu.br

[1] Federal University of Lavras, Lavras, Minas Gerais, Brazil - ORCID: 0000-0002-9975-1626[a], 0000-0002-8379-8008[b] 0000-0001-5698-1838[c], 0000-0001-9326-8716[d]
[2] Universidad Politecnica de Madri, Madrid, Spain - ORCID: 0000-0002-1673-7889[a], 0000-0003-2957-5548[b], 0000-0002-9614-2219[c],

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INTRODUCTION

Leaf-cutting ants are the main forest pests in Brazil (Zanetti et al., 2014). These ants cut large amounts of plant material for the cultivation of their symbiotic fungus in a relationship that was established more than 55 million years ago (Nygaard et al., 2016). The control of these insects is difficult due to their complex social organization, foraging behavior, fungal cultivation, and hygiene habits (Della Lucia et al., 2014).

The most commonly used control method for leaf cutting ants is toxic bait based on sulfluramid (N-ethylperfluorooctane-1-sulfonamide). However, this substance is a precursor of perfluorooctane-1-sulfonate (PFOS), which is extremely persistent in the environment and bioaccumulated in food webs (Löfstedt Gilljam et al., 2016; Zabaleta Lucia et al., 2018). Consequently, restrictions have been imposed on the use of these products (FSC, 2015; OECD, 2018), which has generated discussions about the consequences of this prohibition and the urgency to find new compounds to control leaf-cutting ants (Zanuncio et al., 2016; Lemes et al., 2017).

Thus, the need to develop new active ingredients for the control of leaf cutting ants and the fact that secondary metabolites of plant origin are promising for the control of these pests (Napal et al., 2015; Gomes et al., 2016; Feitosa-Alcantara et al., 2017) justify this research. Among alternative methods, botanical pesticides may provide a sustainable and efficient control of leaf-cutting ants. This is the case, for example, for the metabolites produced by plants of the genus *Psychotria* (Rubiaceae), from which several alkaloids have already been isolated (Lopes et al., 2004; Carvalho Junior et al., 2017) that are secondary metabolites with recognized insecticidal activity (Kato et al., 2018; Wang et al., 2018). To exemplify the potential of *Psychotria* spp., it is noteworthy that the first botanical insecticide for the control of leaf-cutting ants was commercially available in Brazil. This is the granular bait, which has as active ingredients the secondary metabolites from *Tephrosia candida* DC. (Fabaceae) and *Psychotria marcgravii* (A.St.-Hil.) Spreng (Rubiaceae) (MAPA, 2018).

The potential of *Psychotria* spp. for the control of leaf cutting ants has been little explored, thus this work aimed to evaluate the toxicity of alkaloid fractions from *Psychotria hastisepala* Müll. Arg. and *Psychotria leiocarpa* Cham. & Schltdl (Rubiaceae) against *Atta sexdens* Forel, 1908 (Hymenoptera: Formicidae).

MATERIAL AND METHODS

Methanolic crude extracts

Roots, stems, branches and leaves of *P. hastisepala* and *P. leiocarpa* were collected in the Region of Lavras - Minas Gerais (21°13.567; W 044°57.5751), Brazil. The leaves were oven dried at 40 °C for 48 hours, while the other materials were dried for 96 hours. Then, the materials were milled in a Wiley-type mill, giving rise to dry vegetable material with particle sizes smaller than 30 mesh. The dried and ground vegetative materials (50 g) were subjected to static extraction with methanol (MeOH, 300 ml) for 24 hours at room temperature. The obtained mixture was filtered through cotton wool, and the extraction process was repeated eight more times. The liquid phases obtained from each plant material were combined and concentrated on a rotary evaporator to remove the methanol to give a crude methanolic extract.

Preparation of alkaloid fractions

The alkaloid fractions were obtained by the acid-base extraction method, using liquid-liquid sequential partitions, as proposed by Slavíková and Slavík (1966) and cited by Grycová et al. (2007), with modifications. Each crude methanolic extract (5.0 g) was suspended in 100 ml of aqueous sulfuric acid solution 1% (v / v) (H_2SO_4) to give a mixture that was subjected to liquid-liquid partition with diethyl ether (Et_2O) (75 ml). The aqueous phase was separated by decanting and subjected to three more partitions with Et_2O (3 x 75 ml), totaling four partitions. The four phases of Et_2O were combined, giving rise to fraction 1. The acidic aqueous phase, resulting from partitioning with Et_2O was treated with saturated aqueous sodium carbonate solution (Na_2CO_3) to raise the pH to 9, which afforded a residue that was separated by filtration through cotton. The alkaline aqueous phase (pH 9) was partitioned with Et_2O (75 ml x 4). The four new phases of Et_2O were combined, giving rise to fraction 2.

After extraction with Et_2O, sodium hydroxide (NaOH) (0.2 $g{\cdot}ml^{-1}$) was added to the alkaline aqueous phase (pH 9) to achieve a pH of 13 and this was subjected to liquid-liquid partitions with 0.01 $g{\cdot}ml^{-1}$ citric acid solution in Et_2O (75 ml x 4). The four new ethereal phases were combined, giving rise to fraction 3, while the aqueous phase had its pH reduced to 9 by the addition of hydrochloric acid (HCl - 5 $mol.l^{-1}$) to be partitioned with dichloromethane (CH_2Cl_2) (75 ml x 4). The CH_2Cl_2 phases were combined, resulting in fraction 4. The aqueous phase was acidified with HCl (5 $mol{\cdot}l^{-1}$) to pH 6, then 50 ml of saturated potassium iodide solution (KI) was added. The resulting mixture was subjected to liquid-liquid partitions with CH_2Cl_2 (75 ml x 4). The four phases of CH_2Cl_2 were combined to form fraction 5 (Figure 1).

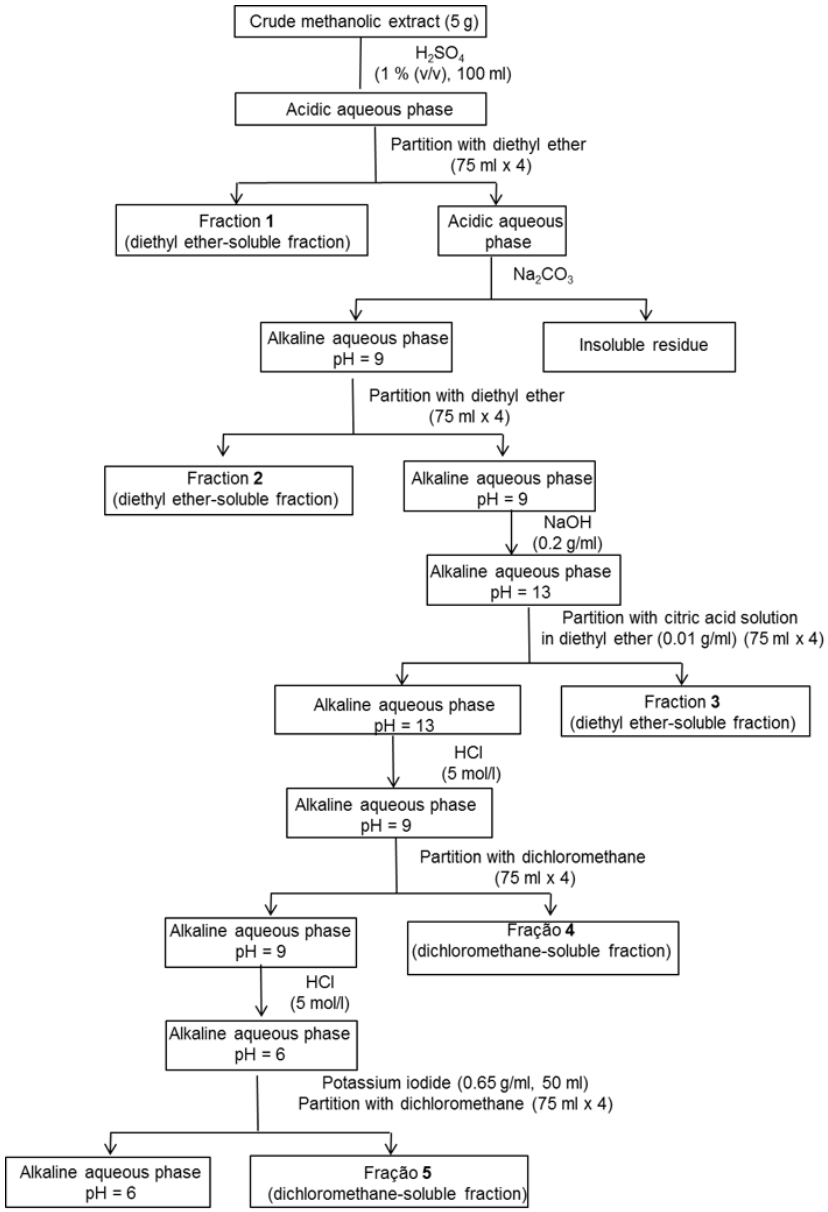

FIGURE 1 Extraction of the alkaloid fractions from the roots, stems, branches and leaves of *Psychotria hastisepala* and *Psychotria leiocarpa* (Rubiaceae).

All liquid fractions were treated with anhydrous sodium sulfate (Na_2SO_4) and concentrated on a rotary evaporator to dryness. The alkaloid fractions from 1 to 5, obtained from each botanical material, were submitted to biological assays with *A. sexdens*, except when the mass obtained from each extraction was insufficient to conduct the bioassay.

Insects

The ants used in the bioassays came from 6-year-old colonies created in laboratory , with a temperature of 21 ± 2 °C, RH of 70 ± 5% and 24-hour scotophase. The anthills were supplied with leaves of *Eucalyptus alba* Reinw. ex-Blume (Myrtaceae), *Hibiscus rosa-sinensis* L. (Malvaceae), *Ligustrum lucidum* W. T. Aiton (Oleaceae) and corn flour and water ad libitum. Ant workers used in the bioassays presented masses between 10 and 20 mg and cephalic capsules between 2.0 and 2.8 mm wide.

Bioassays with *A. sexdens*

The bioassays were conducted according to the adapted methodologies of Bueno et al. (1997) and Gouvea et al. (2011). In each Petri dish, ten workers of *A. sexdens* were placed. The insects were immobilized by exposure to CO_2 for approximately three seconds and before treatment with the fractions previously solubilized

in acetone (10 $\mu g/\mu l$). For the application, 1 μl of each solution was deposited on the back of each ant with a Hamilton syringe. The insects were then transferred to Petri dishes (9 cm in diameter x 2 cm in height) lined with filter paper and containing one piece ($\pm$ 1 cm^2) of artificial diet (Bueno et al., 1997).

The experimental design was completely randomized, and each bioassay was composed of the treatments corresponding to the alkaloid fractions from each plant part and an acetone control, for a total of eight experiments. Six replicates were used per treatment, with each experimental plot consisting of a Petri dish with ten ants. The mortality of the ants was evaluated daily for 21 days, and at the time of the evaluations, the filter paper and diet were changed. The bioassays were maintained in climatic chambers with a temperature of 21 $\pm$ 2 °C, RH of 70 $\pm$ 5% and 24-hour scotophase. The numbers of dead ants per day were submitted to survival analysis using the Weibull distribution, and the adherence of the data to this distribution was verified by means of the Kolmogorov-Smirnov test. Contrast analysis was also performed to verify the similarity between the treatments, aiming at the formation of congeners. In addition, the median lethal time (LT_{50}) was estimated. Statistical analyses were performed with program R (R Development Core Team, 2018), through the Survival package (Therneau, 2017).

RESULTS

The alkaloid fractions from the roots, stems, branches and leaves of *P. hastisepala* reduced the survival of *A. sexdens* (Figure 2). The survival data obtained from the root (D = 0.074074, p = 0.3891), stem (D = 0.036517, p = 0.9715), branch (D = 0.05618, p= 0.628) and leaf fractions (D = 0.073579, p = 0.3932) of *P. hastisepala* adjusted to the Weibull distribution.

The most promising results were obtained when the ants were treated with the root fractions of *P. hastisepala* (χ^2=*83.26*, df=4, p≤0.01). The survival analysis allowed the formation of four congenital groups. The first was formed by fractions 1 and 2, which caused 100% mortality and had TL_{50} of only 2.2 days. The second group was formed by fraction 3, with TL_{50} of 4.1 days and accumulated survival of 2.7%. Fraction 4 formed the third group, with TL_{50} of 4.8 days and survival of 4.5%. The fourth group was formed only by the acetone control (Figure 2A).

In the bioassay with alkaloid fractions of *P. hastisepala* branches, two congener groups were formed, one of which was acetone and the other was composed of fractions 1, 2, 3 and 5 with TL_{50} of 7.1 days and accumulated survival of only 10% (χ^2=*41.54*, df= *5*, p≤0.01) (Figure 2B).

Only fraction 5 from *P. hastisepala* stems was toxic (accumulated survival of 17.6% and TL_{50} = 9 days). Fractions 1, 3 and 4 were statistically similar in performance to the acetone control (Figure 2C) (χ^2=31.26, df=*4* p≤0.01).

Fraction 1 of *P. hastisepala* leaves showed formicidal activity (accumulated survival of 28.4% and TL_{50} = 14.1 days), but fractions 2, 3 and 4 were not toxic (Figure 2 D) (χ^2=19.87, df= *4* p≤0.01).

In the bioassays with alkaloid fractions from the roots (D = 0.10549, p-value = 0.1431), stems (D = 0.056667, p-value = 0.7212), branches (D = 0.048701, p-value = 0.8584) and leaves (0.11986, p-value = 0.13014) of *P. leiocarpa*, the data fit the Weibull distribution. However, fractions from roots (χ^2= 4.16; df =3; p= 0.24), stems (χ^2= 5.5; df=4; p= 0.24), branches (χ^2=6.7; df=4; p= 0.15) and leaves (χ^2=2.5; df= 4, p= 0.64) of *P. leiocarpa* did not cause a reduction in the survival of *A. sexdens* (Figure 3).

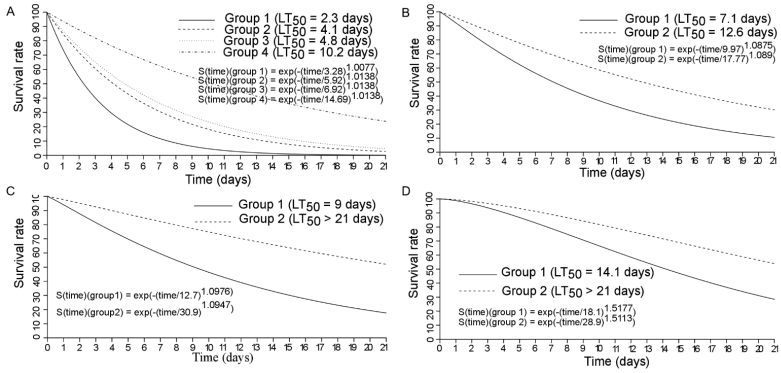

FIGURE 2 Survival analysis of *Atta sexdens* after topical application of alkaloid fractions from *Psychotria hastisepala*. S(t) = exp(-(time/δ) α), δ = shape parameter; α =scale parameter. (A) Alkaloid fractions of *P. hastisepala* roots; group 1: fractions 1 and 2; group 2: fraction 3; group 3: fraction 4; group 4: propanone. (B) Alkaloid fractions of *P. hastisepala* branches; group 1: fractions 1, 2, 3, 5; group 2: propanone, fraction 4. (C) Alkaloid fractions of *P. hastisepala* bark; group 1: fraction 5; group 2: propanone, fractions 1, 3, 4. (D) Alkaloid fractions of *P. hastisepala* leaves; group 1: fraction 1 and group 2: propanone, fractions 2, 3, 4.

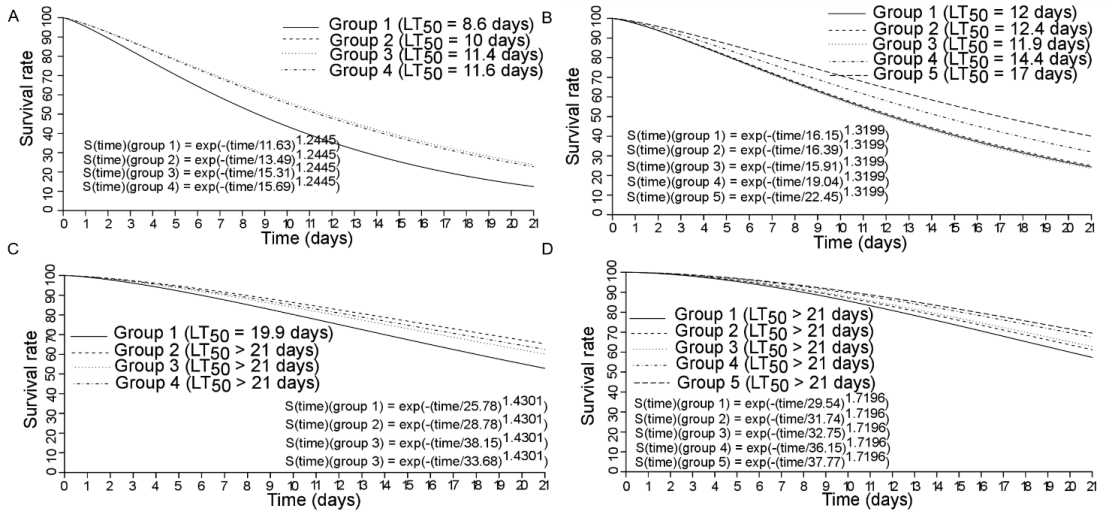

FIGURE 3 Survival analysis of *Atta sexdens* after topical application of alkaloid fractions from *Psychotria leiocarpa*. S(t) = exp(-(time/δ)α), δ = shape parameter; α =scale parameter. (A) Alkaloid fractions of *P. leiocarpa* roots; group 1: fraction 1, group 2: fraction 2; group 3: fraction 4; group 4: propanone. (B) Alkaloid fractions of *P. leiocarpa* branches; group 1: fraction 3; group 2: propanone; group 3: fraction 1; group 4: fraction 2; group 5: fraction 4. (C) Alkaloid fractions of *P. leiocarpa* bark; group 1: fraction 3; group 2: fraction 4; group 3: fraction 2; group 4: propanone; group 5: fraction 1. (D) Alkaloid fractions of *P. leiocarpa* leaves; group 1: fraction 4; group 2: fraction 3; group 3: fraction 1; group 4: fraction 2 and group 5: propanone.

DISCUSSION

In the present work, we investigated the topical insecticidal activity of alkaloid fractions from *P. hastisepala* and *P. leiocarpa* for *A. sexdens*. The most promising results were found for the fractions obtained from the crude methanolic extract of rubiaceous *P. hastisepala* roots.

The Rubiaceae family is known to produce secondary metabolites that are toxic to insects (Chu et al., 2012; Pinto et al., 2012; Tavares et al., 2013; Peres et al., 2017). Among the species of this botanical family, those of the genus *Psychotria* are the most numerous (Davis et al., 2009) diversity, endemism, and taxonomic effort for Rubiaceae are reported, based on queries from a World Rubiaceae Checklist database. Among the species of this botanical family, those of the genus *Psychotria* are the most numerous (Davis et al., 2009) and among the most studied in Brazil (Souza et al., 2013). However, few studies have evaluated the biological activity and conducted phytochemical studies with *P. leiocarpa* and *P. hastisepala*. This is the first work that was carried out aiming to study the formicidal activity of these plant species.

Regarding *P. leiocarpa* it is possible to mention that this species has already been reported as having allelopathic effect, causing inhibition in the germination of lettuce *Lactuca sativa* L. (Asteraceae) seeds (Maraschin-Silva and Aqüila, 2006). In the same sense, was found antiproliferative and genotoxic effects of *P. leiocarpa* infusions on the *Allium cepa* L. (Amaryllidaceae) cell cycle (Lubini et al., 2008). Whereas, for *P. hastisepala* the works developed have focused on ecological aspects such as, flowering and pollination (Silva and Vieira, 2015); reproductive success and genetic diversity (Silva et al., 2014); reproductive phenology and floral morphology (Pereira et al., 2006).

Among the secondary metabolites described from the genus *Psychotria*, the alkaloids stand out (Calixto et al., 2016; Yang et al., 2016; Carvalho Junior et al., 2017). To exemplify the importance of alkaloids in *Psychotria* spp., it is worth mentioning that this class of secondary metabolites has chemotaxonomic value in the genus (Lopes et al., 2004). Thus, in the present work, the extraction of the secondary metabolites was directed to obtain fractions enriched with alkaloids because, in addition to the abundance of these compounds in plants of the genus *Psychotria*, there is a high frequency of insecticidal activity of these metabolites (Ge et al., 2015; Chownski et al., 2016).

Fractions from *P. hastisepala* roots showed greater formicidal activities than those obtained from the other parts of the plant (Figure 2). This can be explained by the fact that plants present quantitative and qualitative variations in the profiles of their secondary metabolites, according to the plant tissue studied (Gobbo-Neto et al., 2017; Jeong and Lim, 2018). It can also be highlighted that, for some secondary metabolites, the biosynthesis site differs from the storage site. Pyridine alkaloids, for

example, are synthesized in the roots and accumulated in the leaves (Shitan et al., 2015).

The most promising results were observed for fractions 1 and 2 from *P. hastisepala* roots (Figure 2A), being these results can be explained due to the extraction method employed in the present study. According to Grycová et al. (2007), the method of extraction of the alkaloids used in the present study causes fraction 1 to contain lipophilic compounds and nonbasic alkaloids that have activity against insects (Wada and Manakata, 1968; Peters, 2016). Fraction 2 can be enriched with quaternary benzophenanthridine alkaloids, protopine alkaloids and tertiary bases soluble in ether. This is in agreement with the fact that alkaloids of these classes have activity against *Lymantria dispar* (Linnaeus) (Lepidoptera: Lymantriidae) (Shields et al., 2008), *Hyphantria cunea* Drury (Lepidoptera: Arctiidae) and *Spodoptera eridania* (Cramer) (Lepidoptera: Noctuidae) (Miller & Feeny, 1983). However, future work is needed to identify the active(s) substance(s).

Alkaloids may act as agonists or antagonists of neurotransmitters (Tang et al., 2008). The action of alkaloids on the nervous system of insects may be related to the fact that, under certain physiological conditions, they present a quaternary nitrogen in their configuration, becoming similar to neurotransmitters (Wink, 2003). In addition, some alkaloids, such as ryanodine and others, affect the muscles of the insects through the connection to the calcium channels of the sarcoplasmic reticulum, causing calcium flow out of the muscle cells, which leads to paralysis and death of the insect.

Although studies of the insecticidal bioactivity of *P. leiocarpa* are scarce, it can be mentioned that in this plant, the indole alkaloid N,β-D-glucopyranosyl vincosamide (Henriques et al., 2004); iridoid glycosides, such as asperuloside and deacetylasperuloside (Lopes et al., 2004), and the cyclotide psyleio A (Matsuura and Fett-Neto, 2013) were detected. The psyleio A, for example, showed insecticidal activity for *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) (Matsuura and Fett-Neto, 2013). However, N,β-D-glucopyranosyl vincosamide did not inhibit herbivory in *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae) (Matsuura and Fett-Neto, 2013). In regard to *P. hastisepala,* no reports of insecticidal activity or phytochemical study were found in the literature.

Fractions 1 and 2 of *P. hastisepala* roots caused mortality in 50% of the population in the time of only 2-3 days, which is very promising (Figure 2A). This time was sufficient for the treated ants to come into contact with untreated ants within their niche, distributing the product to other ants of the colony due to the self-cleaning behavior of these insects.

CONCLUSIONS

In summary, in this work, it was described for the first time that the secondary metabolites produced by *P. hastisepala* are toxic to *A. sexdens*. The fractions 1 and 2, from the roots of this plant, were the most toxic to *A. sexdens*. It is suggested that these fractions are apparently is rich in lipophilic compounds, nonbasic alkaloids, quaternary benzophenanthridine alkaloids, protopine alkaloids and tertiary bases. It is important to note that no reports of insecticidal activity or phytochemical study were found in the literature for *P. hastisepala*.

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