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Bioactivity of *Asclepias curassavica*, *Equisetum* spp. and *Rosmarinus officinalis* Extracts Against Leaf-Cutting Ants

VM Ramos, RG Ferreira Leite, VT Almeida, RS Camargo, JV Souza Cruz, RM Leão, MV Prado, MC Sousa Pereira

Laboratory of Agricultural Entomology, Agronomy Department, College of Agricultural Sciences, University of Western São Paulo [Universidade do Oeste Paulista – UNOESTE], Presidente Prudente-SP, Brazil

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Corresponding author
Vania Maria Ramos
Laboratory of Agricultural Entomology
Agronomy Department
College of Agricultural Sciences
University of Western São Paulo
[Universidade do Oeste Paulista – UNOESTE]
Presidente Prudente-SP, BraSil.
E-Mail: vaniaramos@unoeste.br

ABSTRACT

Chemical control of leaf-cutting ants is widely used, but alternative control with toxic plant extracts is promising. Substances with insecticidal potential extracted from plants have numerous ecological advantages. This study evaluated the insecticidal and/or fungicidal potential of the plants *Asclepias curassavica* (tropical milkweed), *Rosmarinus officinalis* L. (Lamiaceae) (rosemary) and *Equisetum* spp. (horsetail) for control of the leaf-cutting ant *Atta sexdens rubropilosa* Forel, 1908 (Hymenoptera: Formicidae). Forty laboratory-reared colonies of *Atta sexdens rubropilosa* were used. The plants were collected, dried out in a circulating air oven for 48 hours, ground, and macerated in 96° ethanol until exhaustion. After filtration, the products were evaporated under reduced pressure to obtain the ethanolic extracts. Acceptance of the reagent, topical application of the extracts, and application of baits containing 4% of the plant extracts were tested. The results showed that all plant extracts tested negatively influenced the development of the fungus garden. Baits produced with *Asclepias curassavica* caused the highest mortality of the colonies within 7 days. In conclusion, the ethanolic extracts of *Asclepias curassavica*, *Rosmarinus officinalis* and *Equisetum* spp. exhibit insecticidal (contact and ingestion) and fungicidal activity in colonies of the leaf-cutting ant *Atta sexdens rubropilosa*.

Introduction

Leaf-cutting ants of the genera *Atta* and *Acromyrmex* (Hymenoptera: Formicidae) cultivate a symbiotic fungus, *Leucocoprinus gongylophorus* (Heim) (Leucocoprini: Agaricales), that forms the basis of their diet. For this purpose, the ants cut the leaves of different cultured plants and are therefore considered pests in agricultural and forest systems (Mueller et al., 2018). Ant baits are currently the most effective control method indicated for leaf-cutting ants (Britto et al., 2016). However, there has been a growing search for alternative control, especially that involving natural botanical extracts.

Plant extracts with insecticidal and/or fungicidal activity against leaf-cutting ants are widely investigated. The most studied toxic plants are: *Ricinus communis* (Hebling et al., 1996; Bigi et al., 1998; Kitamura et al., 1999; Bigi et al., 2004; Caffarini et al., 2008; Alonso & Santos, 2013), *Sesamum* (Hebling et al., 1991; Bueno et al., 1995; Ribeiro et al., 1998; Peres Filho et al., 2003; Morini et al., 2005; Bueno et al., 2004), *Canavalia ensiformis* (Hebling et al., 2000; Rodriguez et al., 2008; Valderrama-Eslava et al., 2009; Aubad-Lopez, 2011;Varon et al. 2007), *Tithonia diversifolia* (Giraldo-Echeverri, 2005; Castano, 2009) and *Azidarachta indica* (Bigi et al., 2004; Gruber & Valdix, 2003; Herrera, 2009).

Despite the large number of plants studied for the control of leaf-cutting ants, there are three plant species with a promising and unprecedented potential: *Asclepias curassavica*, *Equisetum* sp. and *Rosmarinus officinalis*. The genus *Asclepias*, which comprises about 490 species, is distributed in the Paleotropical, Holarctic and Neotropical regions (Pereira et al., 2004). Some species of this genus

have medicinal properties. For example, *A. curassavica* (Apocynaceae) acts on the central nervous system and is used for the treatment of rheumatism, tumors, inflammation and ophthalmological infections in mammals (Li et al., 2009). High effectiveness of *A. curassavica* extract diluted in ethanol in the control of *Nomophila* sp. caterpillars (Lepidoptera: Noctuidae) has been reported (Costa et al., 2014).

The genus *Equisetum* belongs to the family Equisetaceae and comprises about 30 species. Popularly known as "horsetail", these plants can reach approximately 1 meter in height and are used for therapeutic purposes. Plants of this genus contain high levels of minerals, mainly silicon, and secondary metabolites such as saponins, flavonoids, tannins and alkaloids, which exert beneficial effects on metabolism maintenance and can treat different diseases (Mello & Budel, 2013). Some *Equisetum* species have been used as an alternative pest control system. The extract of this plant confers increased structural stiffness to plant tissues because of the high amount of silicon. This prevents the penetration of fungal hyphae and increases resistance to some phytophagous insects, in addition to influencing the accumulation of phenolic compounds (Bertalot et al., 2010; Mello & Budel, 2013).

Finally, *R. officinalis* L., better known as rosemary, belongs to the family Lamiaceae and is a spice recognized since antiquity for its medicinal effects (Hentz, 2007). The medicinal purposes described are the use of the dry leaves as tea for the treatment of dyspepsia and inflammation (Marsaro-Júnior, 2007). Additionally, rosemary has been used for the control of *Thyrinteina arnobia* leaf-stripping caterpillars (Soares et al., 2011), storage pests (Melo et al., 2011), *Aphis craccivora* aphids (Santos et al., 2011) and bacteria (Ribeiro, 2011), and is also employed because of its antimicrobial activity (Hentz, 2007).

Given the above and considering the harmful effects in insects, we postulate that *A. curassavica*, *Equisetum* sp. and *R. officinalis* have a promising potential for the alternative control of leaf-cutting ants. Therefore, the objective of this study was to evaluate the bioactivity of ethanolic extracts of *A. curassavica*, *Equisetum* sp. and *R. officinalis* on the leaf-cutting ant *Atta sexdens rubropilosa* under laboratory conditions.

Material and Methods

Target species

The experiment was conducted at the Laboratory of Agricultural Entomology, University of Western São Paulo (UNOESTE), Presidente Prudente, São Paulo, Brazil, in a fully climatized room at a temperature of 23.0 ± 1.0°C and humidity of 60% ± 10%.

The *A. sexdens rubropilosa* colonies were collected in the field and stored in plastic containers (1 liter) covered with a plaster layer for moisture balance. The colonies were daily supplied with Acalypha leaves (*Acalypha wilkesiana*). When the fungus reached a minimum volume of 250 ml, two new containers (500 ml) were interconnected at the ends of each colony, one reserved for food and the other used as waste disposal.

Plant extract

For preparation of the extracts, *A. curassavica*, *R. officinalis* and *Equisetum* spp. Were collected in the region of Presidente Prudente - SP. After cleaning and selection of plant parts, leaves and stems were stored in Kraft paper bags and dried out in a circulating air oven at 60°C for 48hours (Table 1). The dried plants were then ground in a Willye knife mill to a particle size of 0.45 mm to obtain a fine powder. The powder was stored in hermetically sealed glass containers at 24°C in the dark until manipulation of the extracts.

The powder of each plant was macerated in 96º ethanol solution and filtered once a day, through a conventional glass funnel, using germination paper as filter paper. After filtration, the flask was filled with 96º ethanol until it covered 4 cm of the powder volume. This procedure was performed until exhaustion in order to obtain the ethanolic extract (Santana et al., 2013).

When a color difference was observed during the filtration process, the whole added solvent was evaporated under reduced pressure in a rotary evaporator, yielding the crude ethanolic extracts of *A. curassavica*, *R. officinalis* and *Equisetum* spp.

Fabrication of the baits

The baits were fabricated by mixing ground citrus pulp (300 g), carboxymethylcellulose (0.8 g), vegetable oil (2.0 g), distilled water (300 ml), 96º ethanol (240 ml), and the plant extract at a proportion of 4% (w/w) (Ramos et al., 2006).

After grinding the pulp, carboxymethylcellulose, oil and water were added and the mixture was homogenized. The crude extract was dissolved in ethanol and added to the mixture in an autoclaved glass container, forming a paste that could be molded. The control treatment was prepared without the plant extract. The pasty mixture was inserted and compacted in a 3-ml syringe with the tip cut off and arranged on a tray covered with aluminum paper, forming strands of approximately 5 cm that were cut into smaller pieces (pellets). Finally, the pellets were dried in an oven at approximately 50ºC for 24 hours.

Table 1. Denomination and origin of the plants used for preparation of plant extracts.

Scientific name	Common name	Family	Parts used	Origin
Asclepias curassavica	Tropical milkweed	Asclepiadaceae	Leaves and stems	Presidente Prudente - SP
Rosmarinus officinalis	Rosemary	Lamiaceae	Leaves and stems	Paraguaçú Paulista - SP
Equisetum spp.	Horsetail	Equisetaceae	Leaves and stems	Presidente Prudente - SP

Bioassay for testing the repellency or rejection of plant extracts by worker ants

This assay aimed to evaluate if the solvent used for preparation of the baits influences bait loading, i.e., if it promotes repellency or rejection. Two treatments were compared to determine acceptance of the material. In the first treatment, 5-mm filter paper discs were immersed in 96º ethanol and immediately rolled in ground citrus pulp to increase their attractiveness. In the control treatment, the filter paper discs were immersed in distilled water before rolling in citrus pulp powder.

The experimental unit was set up as the foraging chamber of a colony. We used a total of 30 experimental units, divided into fifteen colonies used as replicates per treatment. For each replicate (colony), 20 dry filter paper discs were simultaneously supplied, totaling 300 discs per treatment. Loading, incorporation and return of the discs in the waste chamber were evaluated after 24 hours according to Ramos (2005).

Bioassay for testing topical action of the plant extracts on workers

The aim of this assay was to evaluate ant mortality resulting from contact application of the plant extracts. Since ants exhibit a self-grooming and grooming behavior, they were used individually, ensuring that the effect of the extracts was not due to ingestion.

The experimental unit was set up as a plastic container (75 ml) with plaster covering the bottom in order to maintain humidity, and one worker added to it. Worker ants of medium size were removed randomly from healthy colonies and placed inside containers. A piece of fungus (0.5 cm^3) was placed inside each container, which served as food for the ants. The fungus was always changed when it had lost its nutritional value as demonstrated by a decrease in its volume and color change. The experiment consisted of 5 treatments: ethanolic extract of *A. curassavica* (4%), ethanolic extract of *R. officinalis* (4%), ethanolic extract of *Equisetum* spp. (4%), ethanol, and distilled water. For each treatment, 30 plastic containers with one ant were used as replicates, totaling 150 replicates. Each worker received an application of 0.5 µl of the respective treatment on its pronotum. The evaluations of worker mortality were conducted at 2, 8, 12, 24, 48, 72, 168 and 384 hours after extract application.

Bioassay for testing the effect of the baits containing the plant extracts on colonies

This assay aimed to evaluate the effect of baits containing the plant extracts on *A. sexdens rubropilosa* colonies. The experimental unit was comprised of a colony containing both foraging and waste chambers, and all colonies were the same age (approximately 1 year) and same size (500 mL fungus garden). Forty colonies were randomly assigned into four treatments, which led to 10 replicates per treatment. The following treatments were applied: citrus pulp baits containing 4% ethanolic extract of *A. curassavica*, *R. officinalis* and *Equisetum* spp. As well as ethanol as control.

At the beginning of the experiment, all colonies had their residues discarded and no leaves were supplied for 48 hours. In addition, the initial volume of the fungus garden of each replicate was determined as the proportion of fungal volume in relation to the total volume of the container. Each replicate received 1 g of bait (pellets) in their foraging chamber for 7 consecutive days. Twenty-four hours after each supply, the number of pellets carried by the workers, pellets incorporated into the fungus garden, and pellets returned to the waste chamber was counted. Additionally, the presence of fungus discarded in the waste and the number of dead ants were daily evaluated. In order to avoid interference with the effect of the treatments on the colonies, *A. wilkesiana* leaves was also daily supplied as foraging substrate.

The volume of the fungus was evaluated on days three and seven after application of the treatments, determining whether the volume of the fungus garden remained the same or decreased as a result of application of the treatments. Ant mortality was scored as 0, 25, 50 and 100. Score 0 represents the natural mortality of ants, corrected for the control and daily observed with minimum numbers as a function of the normal life cycle of the ants. Score 25 corresponds to the mortality of approximately 25% of ants present in the colony, score 50 to 50%, and score 100 to complete mortality of workers.

Statistical analysis

First, the data were submitted to the Shapiro-Wilk normality test. Since the data were not normally distributed, the behavior results were submitted to nonparametric analysis. The survival curves of workers were compared by the log-rank test. Bait loading, bait incorporation, fungal volume and worker mortality in the colonies were analyzed by the Kruskal-Wallis test. If significance was observed, each variable was compared by the Student-Newman-Keuls post-test, adopting a level of significance of 5%. Separate analyses were performed at 24 hours, 72 hours, and 7 days.

Results

Bioassay of repellency and rejection of the plant extracts

The filter paper discs of the two treatments were equally carried out by workers and incorporated into the fungus garden (100% loading and incorporation). These results suggest that citrus pulp has a superior odor to ethanol or that ethanol evaporates completely and leaves no residues. This finding confirms that ethanol does not cause repellency or rejection by ants and can be used in the formulation of toxic baits containing plant extracts without interfering with the result of the final product.

Bioassay of the topical action of the plant extracts on workers

The results showed a strong effect of the plant extracts on worker mortality, particularly until 168 hours (7 days) after topical application (Figure 1). The *A. curassavica* extract was the most promising, with a cumulative mortality of 70 to

90% between 48 and 72 hours after application of the extract. Comparison of worker survival curves showed a significant difference between ethanol (control) and the *Equisetum* spp. (log-rank test, X^2=4.56, d.f.=1, p<0.05), *A. curassavica* (log-rank test, X^2=61.01, d.f.=1, p<0.001) and *R. officinalis* (log-rank test, X^2=9.91, d.f.=1, p<0.05) extracts. A significant difference was also detected between the survival curves of *A. curassavica* and *Equisetum* spp. (log-rank test, X^2=12.42, d.f.=1, p<0.001) and *A. curassavica* and *R. officinalis* (log-rank test, X^2=39.18, d.f.=1, p<0.001). There was no significant difference between the survival curves of workers receiving water and ethanol (log-rank test, X^2=0.0023, d.f.=1, p>0.05).

Bioassay on the effects of the baits containing the plant extracts on colonies

The results showed a deleterious effect of baits containing plant extracts on colonies of leaf-cutting ants (Figure 2). Regarding bait loading, a significant difference was observed between treatments at 24 hours (Kruskal-Wallis test, H=35, d.f.=3, p<0.001), 72 hours (Kruskal-Wallis test, H=39, d.f.=3, p<0.001), and 7 days (Kruskal-Wallis test, H=38, d.f.=3, p<0.001). As can be seen in Figure 2-A, loading declined after 24 and 72 hours. The control baits (ethanol) and baits containing the *R. officinalis* extract did not differ significantly from one another, but differed from those containing the *A. curassavica* and *Equisetum* spp. Extracts. On day 7, lower loading by workers was observed for baits containing *A. curassavica* extract, which differed from the other treatments (Figure 2-A).

Incorporation of baits into the fungus garden also differed between treatments, with the observation of a significant difference at 24 hours (Kruskal-Wallis test, H=34, d.f.=3, p<0.001), 72 hours (Kruskal-Wallis test, H=37.5, d.f.=3, p<0.001), and 7 days (Kruskal-Wallis test, H=36, d.f.=3, p<0.001). A reduction in the rate of bait incorporation was observed at 24 and 72 hours (Figure 2-B). The control baits (ethanol) and baits containing the *R. officinalis* extract did not differ significantly from one another, but differed from those containing the *A. curassavica* and *Equisetum* spp. Extracts. On day 7, baits containing the *A. curassavica* and *Equisetum* spp. Extracts were less incorporated by workers, differing from the other treatments (Figure 2-B).

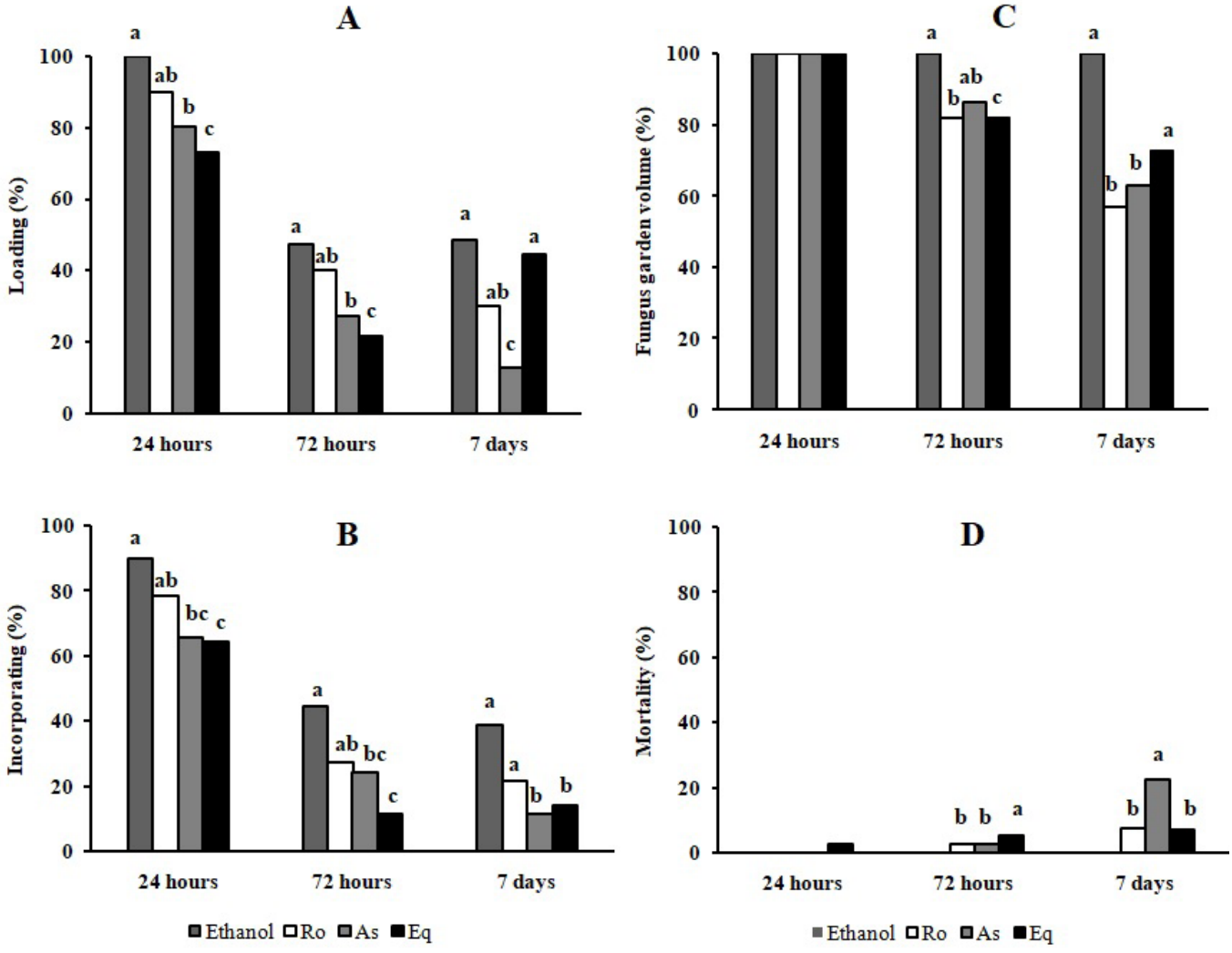

Figure 1. Survival curves of leaf-cutting ants over a period of 384 hours. Eq (*Equisetum* spp. extract), As (*Asclepias curassavica* extract), Ro (*Rosmarinus officinalis* extract), water, ethanol.

A reduction in fungus garden volume was observed for each treatment (Figure 2-C), with a significant difference at 72 hours (Kruskal Wallis test, H=34, d.f.=3, p<0.001) and 7 days (Kruskal-Wallis test, H=38, d.f.=3, p<0.001). The baits containing the *A. curassavica* and *Equisetum* spp. Extracts promoted the greatest reduction in the volume of the fungus garden (Figure 2-C).

The baits containing plant extracts also caused significant mortality of workers, with significant differences between treatments at 24 hours (Kruskal-Wallis test, H=22,29, d.f.=3, p<0.001), 72 hours (Kruskal-Wallis test, H=38,29, d.f.=3, p<0.001), and 7 days (Kruskal-Wallis test, H=38,36, d.f.=3, p<0.001). At 24 hours, high mortality was observed for colonies treated with baits containing the *R. officinalis* extract (Figure 2-D). On day 7, high worker mortality was found in colonies treated with the *A. curassavica* extract (Figure 2-D).

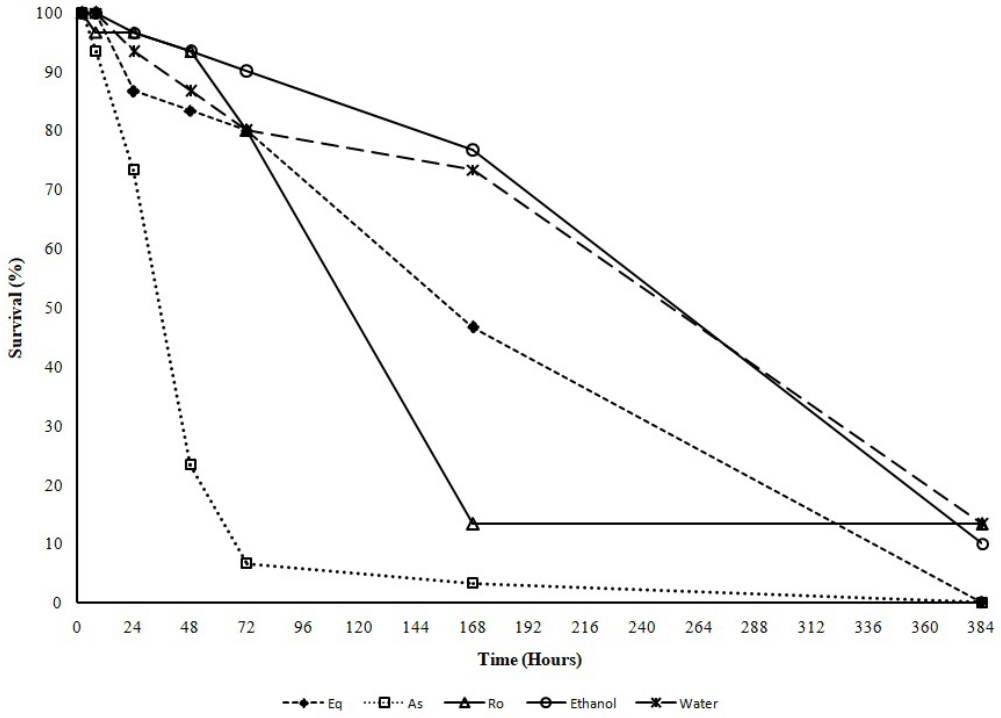

Figure 2. Percentage of loading and incorporation of baits (A and B), fungus garden volume (C), and worker mortality (D) in *Atta sexdens rubropilosa* colonies under laboratory conditions. Different letters above the columns indicate a significant difference by the Student-Newman-Keuls post-test (p<0.05). The same letters indicate the absence of a difference.

Discussion

The present study results show significant insecticidal and fungicidal activity of the ethanolic extracts of *A. curassavica*, *Equisetum* sp. and *R. officinalis* against *A. sexdens rubropilosa* colonies (Figures 1 and 2).

The topical application of the extracts to workers significantly reduced survival (Figure 1), especially the *A. curassavica* extract. Regarding the insecticidal activity of toxic plants, the hexane and dichloromethane fractions of leaf extracts of *R. communis*, applied topically, also exhibited insecticidal activity (Bigi et al., 1998). Fatty acids and ricin were found to be toxic to *A. sexdens rubropilosa* workers at concentrations of 0.2 and 0.4mg.ml^{-1} (Bigi et al., 2004). Contact activity has also been reported for extracts of Amazon plants (*Banara guianensis*, *Clavija weber baueri*, *Mayna parvifolia*, *Ryania speciosa*, *Spilanthes oleraceae* and *Siparuna amazônica*) against *A. sexdens*, *A. laevigata* and *Acromyrmex subterraneus molestans* at a concentration of 5mg.ml^{-1} (Gouvea et al., 2010). In addition, compounds such as terpenoids, caryophyllene epoxide, nerolidol and kolaenol isolated from *Hymenaea courbaril*, *Melampodium divaricatum* and *Vismia bacciferae* exhibited insecticidal activity when workers were supplied with an artificial diet containing these compounds (Howard et al., 1988). Feeding artificial diets, Boulogne et al. (2018) observed insecticidal activity of the seed extract of *Mammea americana* against *Ac. octospinosus* workers whose lethal concentration was close to that of Fipronil (0.03g.kg^{-1}). The authors attributed this effect to the significant presence of alkaloids, phenolic compounds, and terpenes in this plant.

The *Equisetum* spp., *A. curassavica* and *R. officinalis* extracts clearly exhibited insecticidal activity by direct contact and ingestion, as demonstrated by worker mortality after topical application and the presence of dead workers in colonies that received the baits with the extracts (Figure 1, Figure 2-D). Many compounds of botanical extracts exhibit this feature. For example, the crude seed extract of *A. indica* caused significant ingestion and contact toxicity in *A. sexdens* workers (Santos Oliveira et al., 2006). In *A. cephatoles*, the

extract of *Tithonia diversifolia*, at a concentration of 1.5 $ml.l^{-1}$, was efficient as topical application and when ingested (Castano, 2009). Substances purified from *Helietta puberula*, including anthranilicacid, kokusaginine and dictamnine, provided in artificial diets were toxic to *A. sexdens* workers (Almeida et al., 2006). The alkaloid, 5-methoxy-canthin-6-one, isolated from the plant *Simarouba versicolor* was also toxic to *A. sexdens* workers (Penaflor et al., 2009). Furthermore, Boulogne et al. (2011) found that topically applied *M. americana* and *Nicotiana tabacum* extracts were toxic to workers, and *Nerium oleander* extract exhibited a delayed action, although many fractions were repellent to ants.

Another interesting topic to be addressed refers to the chemical compounds present in the extracts that are toxic to leaf-cutting ant workers. The seed oils of *R. communis* a nd *Jatropha curcas* were toxic by ingestion at a concentration of 10 and $30mg.ml^{-1}$, respectively, and by direct contact at a concentration of 0.1 and $0.2mg.ml^{-1}$ (Alonso & Santos, 2013). The authors suggested that ricinoleic, oleic and linoleic acids are the toxic agents of *Ricinus* oil. Another compound is coumarin, identified in extracts of rue (*Ruta graveolens*), jimsonweed (*Datura estramonium*), *Cordia verbenaceae*, peppermint (*Mentha pitanperita*), goat weed (*Ageratum conyzoides*) and tropical apricot (*M. americana*), which is a potential insecticide for leaf-cutting ants (Araujo et al., 2008; Boulogne et al., 2018). *Equisetum* sp. plants are known for their high content of minerals, especially silicon, and secondary metabolites such as saponins, flavonoids, tannins and alkaloids (Mello & Budel, 2013). One of the main active compounds of *A. curassavica* is the glycoside asclepiadin (Costa et al., 2014; Carvalho et al., 2009), which causes toxicity in mammals (Costa et al., 2014). *R. officinalis* contains different active compounds found in the essential oil prepared from the leaves and flowers (Hentz, 2007). These compounds are probably toxic to leaf-cutting ants since they caused the death of workers.

The mode of action of most of these compounds is unknown, probably because of the difficulty in performing physiological studies. Few researchers have studied the mode of action of compounds from toxic plants. For example, bullatacin is a compound isolated from plants of the family Annonaceae, which has a great insecticidal potential. This compound strongly inhibits cellular respiration, exerting an antagonistic effect on the electron transport in mitochondria, with a specific action on complex I (Ahammadsahib et al., 1993). However, we do not know about the continuation of that research. Other compounds with a neurophysiological focus are being studied; for example, silphinenes, compounds extracted from the aerial part of *Senecio palmensis*. This tricyclic sesquiterpene acts as an antagonist of the ɤ-aminobutyricacid (GABA) system, more specifically on chloride receptor channels (Bloomquist et al., 2008). Another compound with neurotoxic activity is the monoterpenoid pulegone-1, 2-epoxide, isolated from *Lippiasteochadifolia* (Grundy & Still, 1985). The authors demonstrated that this compound acts like carbamates, irreversibly inhibiting the enzyme acetylcholinesterase. Little information is available on extracts of *A. curassavica*, *Equisetum* sp. and *R. officinalis* and their active compounds, especially the mode of action of these compounds.

Expressive fungicidal activity was observed in the experiments (Figure 2-C), demonstrated by the drastic reduction in the volume of the fungus garden of the colonies. Synthetic fungicides such as cycloheximide have the same effect (Sousa et al., 2017). Previous studies have shown that natural botanical extracts promote a decrease in the fungus garden of leaf-cutting ants. After 6 weeks of treatment, *M. americana* seed extract caused total decline of *Ac. octospinosus* colonies and the symbiotic fungus garden was completely infested with competitors (Boulogne et al., 2018). Pagnocca et al. (1990) found that *Sesamum indicum* extract caused a decrease in the growth of *Leucocoprinus gongylophorus*, but the authors did not identify the compounds of this extract. In a subsequent study, these authors obtained the same result for lignans of *Virola sebifera* and *Otoba parvifolia* (Pagnocca et al., 1996). Lignans are common components of plants. More than 500 compounds that have antitumor, antimitotic and antiviral activity have been described (Macrae & Towers, 1984). Pagnocca et al. (1996) concluded that lignans are the most potent compounds against the symbiotic fungus and ants therefore do not cut *V. sebifera* leaves.

Sesamin exerted a fungicidal effect at a concentration of $70\mu g.ml^{-1}$ (Bueno et al., 2004). This result disagrees with the findings of Ribeiro et al. (1998) who demonstrated complete inhibition of the fungus at a low concentration ($2.5\mu g.ml^{-1}$). Bigi et al. (2004), studying the activity of *R. communis* extract, reported that fatty acids are important compounds against the fungus of leaf-cutting ants. However, it is not known which fatty acid was responsible for inhibition of the fungus. In a chromatographic analysis of *S. indicum* extracts, Ribeiro et al. (1998) purified substances with fungicidal activity, including tetradecadonoic, hexadecanoic, 9, 12, 15-octadecatrienoic, octadecanoic, icosanoic and docosanoic acids, but an attempt to isolate the substances was unsuccessful. A similar result was found for the fatty acids and crude extract of *Canavalia ensiformis* (Monteiro et al., 1998). Based on this knowledge, Castro Faria and Sousa (2000) added *S. indicum* seeds to the culture medium of the symbiotic fungus of *Acromyrmex* leaf-cutting ants and concluded that the seeds affect fungal growth.

The dichloromethane and hexane fractions of *Cipadessa fruticosa* and *Cedrela fissilis* extracts exerted an inhibitory effect on the symbiotic fungus of leaf-cutting ants (Bueno et al., 2005; Leite et al., 2005). Almeida et al. (2006) isolated 6 substances from *Helietta puberula*, including anthranilicacid, kokusaginine and dictamnine, which exhibited a strong inhibitory effect on the symbiotic fungus of *A. sexdens*. These alkaloids are a promising source of new substances for the control of leaf-cutting ants. Similar results were obtained for another alkaloid, 4, 5-dimethoxy-canthin-6-one, isolated

from *S. versicolor* extract (Penaflor et al., 2009). Caffeine (1, 3, 7-trimethylxanthine) was also used to evaluate the *in vitro* growth of the symbiotic fungus of *A. sexdens rubropilosa* and concentrations of 0.1 and 0.5% caused fungal death in the laboratory (Miyashira et al., 2011). Taken together, these results show that the synergistic action of substances can affect the development of the fungus garden of leaf-cutting ants, similar to the results of the present study using *A. curassavica*, *Equisetum* sp. and *R. officinalis*.

In conclusion, the ethanolic extracts of *A. curassavica* (tropical milkweed) *R. officinalis* (rosemary) and *Equisetum* spp. (horsetail) exhibit insecticidal (by contact and ingestion) and fungicidal activity in colonies of the leaf-cutting ant *A. sexdens rubropilosa*. However, further assays using different doses and bait application methods are necessary to obtain definitive results that can be recommended for the field.

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