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Short Communication

Chemical analysis and hydroalcoholic extract activity of ferns on the mutualist fungus of leafcutter ants

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Abstract

Leafcutter ants are among the most important agricultural and forest pests in the Neotropical region, given that they use plant matter as substrate for the growth of their mutualist fungus *Leucoagaricus gongylophorus*. Plant-based insecticides and fungicides have shown potential for controlling these ants. The present study assessed the hydroalcoholic extract activity of four fern fronds (dry and rainy periods) on the mutualist fungus of leafcutter ants. Fungal mycelium suspensions were seeded on the surface of tubes containing fern extracts at a concentration of 5 mg/mL and 100 µg/mL. The chemical profile of the extracts was analyzed by thin layer chromatography (TLC). Extracts (dry period) at a concentration of 5 mg/mL inhibited the growth of the fungus: *Macrothelypteris torresiana* and *Dicksonia sellowiana* (less than 20%), *Niphidium crassifolium* (approximately 40%), *Parapolystichum effusum* extract (100%). None of the extracts (dry and rainy periods) inhibited fungal growth at a concentration of 100 µg/mL. The chemical profile analysis of the extracts indicated the presence of beta-sitosterol, friedelinol, rutin, and kaempferol. The crude extracts of *P. effusum* and *N. crassifolium* were the most promising in future formulations of antifungal products. Thus, ferns are potential plants in the search for environmentally-friendly substances for sustainable agriculture.

Key words: antifungals, biological interactions, *Leucoagaricus gongylophorus*, pteridophytes.

Resumo

Formigas cortadeiras estão entre as pragas agrícola e florestal mais importante da região Neotropical, uma vez que utilizam material vegetal como substrato para crescimento de seu fungo mutualista *Leucoagaricus gongylophorus*. Inseticidas e fungicidas de origem vegetal têm mostrado potencial para o controle dessas formigas. O presente estudo avaliou a atividade de extratos hidroalcóolicos de frondes de quatro espécies de samambaias (períodos seco e chuvoso) sobre o fungo mutualista de formigas cortadeiras. Suspensões do micélio fúngico foram semeadas na superfície dos tubos contendo extratos das samambaias na concentração de 5 mg/mL e 100 µg/mL. O perfil químico dos extratos foi analisado por cromatografia em camada delgada (CCD). Extratos (período seco) na concentração de 5 mg/mL inibiram o crescimento do fungo: *Macrothelypteris torresiana* e *Dicksonia sellowiana* (menos de 20%) *Niphidium crassifolium* (aproximadamente 40%), *Parapolystichum effusum* (100%). Os extratos (períodos seco e chuvoso) na concentração 100 µg/mL não promoveram inibição. A análise do perfil químico dos extratos das quatro espécies indicou a presença de beta-sitosterol, friedelinol, rutina e canferol. Os extratos brutos de *P. effusum* e *N. crassifolium* foram os mais promissores para futuras formulações de produtos antifúngicos. Assim sendo, as samambaias são plantas potenciais na busca de substâncias com menor impacto ambiental para uma agricultural sustentável.

Palavras-chave: antifúngicos, interações biológicas, *Leucoagaricus gongylophorus*, pteridófitas.

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Leafcutter ants from the Attini tribe, genus *Atta*, are the most important agricultural and forest pests in the Neotropical region (Cherrett 1986; Della Lucia 2003; Leal *et al.* 2014), with the species *Atta sexdens rubropilosa* (Forel 1908) one of the most serious pests in Brazil (Della Lucia 2003). Synthetic insecticides are the most frequently used to control these ants. According to Boulogne *et al*. (2012) and Nickele *et al.* (2013), this method is also the most harmful to the environment and human health, in addition to being nonspecific. Due to the considerable ecological and economic impact caused by leafcutter ants, the discovery of new environmentally less aggressive formicides and/or fungicides is needed. It is known that one of the most effective ways to combat leafcutter ants is by eliminating their symbiont fungus, and increasingly more studies on the antifungal activity of plants are being conducted (Pagnocca *et al.* 1990; Rezende *et al*. 2007; Souza *et al.*2011; Pereira 2012; Oliveira 2015). New strategies have been used, especially the study of possible plant insecticides (Boulogne *et al*. 2012; Nickele *et al*. 2013) and fungicides (Hebling *et al*. 2000; Bigi *et al.* 2004; Bueno *et al.* 2005).

Leafcutter ants are described by Aylward *et al.* (2013) as a prime example of insect-plant interaction, since cultivation of the fungus *Leucoagaricus gongylophorus* (A. Møller) Singer (Basidiomycota, Agaricaceae) on fresh plant matter guarantees a nutrient supply to the ants that would otherwise be unavailable. They are considered obligatory fungivores because their symbiont fungus is the only food source for their larvae and the main nutritional source for adults (Quinlan & Cherret 1979; Cherret 1986; Nickele *et al.* 2013). The symbiont fungus of leafcutter ants (*L. gongylophorus*) contains hyphal swellings on its extremities called gongylidia, which accumulate nutrients and digestive enzymes (Howard 1988). This specialization demonstrates the high degree of fungal domestication by the ants (Nickele *et al.* 2013).

For self-protection, many plants have mechanisms that prevent or at least minimize herbivory and infection by pathogenic organisms. These defenses can be mechanical, structural or chemical (Taiz & Zeiger 2004). Terpenoids, which stand out for their abundance and diversity, act in the chemical defense of plants, exhibiting several biological activities, including insecticide, repellent, anti-inflammatory, analgesic, anticholinesterase, and antimicrobial action (Dewick 2009; Hichri *et al.* 2003; Pinto *et al*. 2008; Soldi *et al.* 2008; Fernandes *et al*. 2011; Fürstenberg-Hägg *et al.* 2013). Phenolic compounds are involved in chemical defense against herbivores and pathogens, with tannins standing out for their antimicrobial and antioxidant properties (Santos & Mello 1999; Johann *et al*. 2007; Pessuto *et al.* 2009). Lima *et al*. (2022) reported that terpenoids and phenolic substances are associated with the insecticidal/repellent activities of ferns and lycophytes.

Insects may exhibit pharmacophagous behavior, whereby they consume beneficial non-nutritional substances (for example, secondary metabolites or drugs) present in the plants of their diets (Bopré 1984; Costa-Neto 2012). Bopré (1984) cites the example of adult butterflies of the Danainae subfamily (family Nymphalidae) that actively collect pyrrolizidine alkaloids from plants to use in defense and pheromone production.

However, when behavior goes beyond pharmacophagy, that is, when there is self-medication, it is called zoopharmacognosy (Costa-Neto 2012). According to Shurkin (2014), zoopharmacognosy is the innate ability of animals to detect therapeutic components in plants. According to the author, although it remains unknown exactly how much inherited learning or knowledge is involved in this recognition, the chemical composition of plants is clearly the guiding characteristic in this process. Melliferous bees and wood ants that line their nests with resin to combat bacteria are good examples of zoopharmacognosy in insects (Shurkin 2014).

According to Santana (1988), leafcutter ants select plant matter, distinguishing between species of the same genus and even same species with different origins. The chemical and physical factors of plant matter are parameters that directly influence symbiont fungal growth (Cherrett 1972; Stradling 1978; Fowler & Stiles 1980) and ant selection (Santana 1988). Bioassays conducted by Mehltreter & Valenzuela (2012) revealed that leafcutter ants [*Atta mexicana* (Smith 1858)] preferred some types of fern species, and totally rejected or scarcely used others. Studies carried out by Howard (1988) suggest that ants select cut leaves according to secondary metabolite composition, choosing only those that they and their fungi can tolerate.

In this respect, we can raise the hypothesis that leafcutter ants may select plant matter to take to the nest based on the presence or not of substances with antifungal action in plants, which may interfere in the growth of its symbiotic fungus. With a view to testing this hypothesis, the hydroalcoholic extract action of the fronds (leaves) of four fern species was assessed in relation to the symbiont fungus *Leucoagaricus gongylophorus*. The qualitative profile of terpenoids and phenolic substances in hydroalcoholic extracts is also presented, as well as the effect of dry and rainy periods on the chemical profile of these extracts.

Four fern species were selected, using as reference the preference bioassays of the leafcutter ant *Atta mexicana* (Smith 1858) (Mehltreter & Valenzuela 2012). The species *Dicksonia sellowiana* Hook. (Dicksoniaceae), *Macrothelypteris torresiana* (Gaudich.) Ching (Thelypteridaceae), *Parapolystichum effusum* (Sw.) Ching (Dryopteridaceae), and *Niphidium crassifolium* (L.) Lellinger were ultimately used in the aforementioned study (Fig. 1).

The species were collected in October 2016 (end of the dry period) and April 2017 (end of the rainy period) (Barbieri 2005). *Dicksonia*

Figure 1 – a-d. Ferns used for antifungal assessment – a. *Dicksonia sellowiana*; b. *Niphidium crassifolium*; c. *Macrothelypteris torresiana*; d. *Parapolystichum effusum*. Photos: Marcelo Guerra Santos.

sellowiana (22°22'35.9"S, 44°45'38.1"W), *Macrothelypteris torresiana* (22°26'43.6"S, 44°36'37.7"W), and *Niphidium crassifolium* (22°25'43"S, 44°37'10.4"W) were collected in the Itatiaia National Park, Brazil, and *Parapolystichum effusum* (22°52'01.3"S, 42°41'15.1"W) at the Cachoeira do Espraiado, municipality of Maricá, Rio de Janeiro state, Brazil. Collections were authorized for scientific activities, through authorization no. 53534-2, issued on 11/28/2016 by the Instituto Chico Mendes de Conservação da Biodiversidade-ICMBio (Chico Mendes Biodiversity Conservation Institute).

Part of the botanical material was herborized according to the techniques described by Pietrobom *et al.* (2023), and all the exsiccates were deposited in the Faculdade de Formação de Professores herbarium of the Universidade do Estado do Rio de Janeiro (RFFP), *D. sellowiana* (RFFP20814), *M. torresiana* (RFFP21908) *N. crassifolium* (RFFP21909), *P. effusum* (RFFP21910).

The fronds were oven dried separately at 50 °C. After maceration they were weighed and submitted to extraction with 96% ethanol (v/v), using the static maceration technique, with daily agitation, for 15 days. In order to obtain crude extract, the ethanol was removed by evaporation, using a rotary evaporator at an average temperature of 40 °C. At the end of the process, the extracts were stored at ambient temperature. The entire procedure was conducted at the Laboratório de Tecnologia de Produtos Naturais (LTPN) of the Universidade Federal Fluminense (UFF), Pharmacy School. In a preliminary test, crude plant extracts (dry period) were solubilized using absolute ethanol at a ratio of 1:2, totaling 50 mg/mL. The preliminary test was a screening of the fungicide activity in high concentrations of extracts. Each extract was submitted to ultrasound for three minutes, guaranteeing total solvent solubilization. In a second test, the extracts (dry and rainy periods) were solubilized in methanol, reaching a concentration of 1 mg/mL.

The FF 2006 strain of basidiomycete fungus *Leucoagaricus gongylophorus* was obtained from the Microbiology Laboratory of the Center for Social Insect Studies (CEIS) of the Universidade Estadual Paulista Júlio de Mesquita Filho (UNESP). In line with the methodology described by Pagnocca *et al*. (1990), the fungus was seeded on a medium containing glucose (10 g/L), sodium chloride (5 g/L), bacto-peptone (5 g/L), malt extract (10 g/L) and agar (15 g/L) (Medium A) and placed in tubes. Two controls were prepared concomitantly in each test, one with only medium A and another containing medium A added to the ethanol solvent. Each tube received 1 mL of solubilized extract, added with 9 mL of medium A, totaling a final concentration of 5 mg/mL (preliminary test) and 100 µg/mL (second test). The experiment used 30-day-old *L. gongylophorus* fungus colonies, isolated in five 250x25 mm tubes containing the culture medium. All the mycelium in the tubes was transferred to the Potter homogenizer containing 3 mL of 0.1 % peptone water, creating a suspension. The suspension (containing about 3 or 4 mg of dry weight/mL) was seeded on the inclined surface of the medium and incubated for twenty days at 25 ^{o}C. Given that *L. gongylophorus* does not exhibit radial growth, its growth was visually assessed considering the basis and density of mycelial growth, 30–35 days after incubation. Fungal growth inhibition was assessed against controls, and characterized on the following scale: 0%–20%; ≥21%–40%, ≥41%–60%, ≥61%–80% and ≥81%–100% (Pagnocca *et al.* 1990). The experiments were carried out in triplicate in both periods.

Chemical profile analysis of the extracts was conducted by thin layer chromatography (TLC), using chemical markers for terpenoids (beta-sitosterol and fridelinol) and phenolic substances (rutin and kaempferol). Four µL samples of extracts from the dry and rainy periods were applied, diluted in methanol (50 mg/mL), on TLC silica gel F254 plates (SILICYCLE inc.). For the terpenoid assessment plate, a mobile phase composed of hexane:ethyl acetate (7:3) and vanillin-sulfuric reagent were used, with reading performed in a darkroom at a UV wavelength of 365 nm. For the phenolic compound assessment plate, the mobile phase consisted of ethyl acetate:formic acid:acetic acid:water (100:11:11:26), and the reagents were diphenyl-boryloxyethylamine reagents (NP) followed by polyethylenoglycol (PEG), read in a darkroom at a UV wavelength of 254 nm.

The similarity of TLC plates in analyzing the terpenoids and phenolic substances of fern species (dry and rainy periods) was determined by assessing the retention factors of the substances contained in the extracts. The Sørensen-Dice coefficient was used to measure the distance between the species and periods (dry and rainy). Dendograms were constructed using cluster analysis of the UPGMA algorithm (Unweighted Pair Group Method with Arithmetic Mean), where the groups are clustered based on the average distance between all the members of both groups (Gotelli & Ellison 2011), using PAST (Paleontological Statistics) program, version 3.10 (Hammer *et al.* 2001).

Hydroalcoholic extract of the fronds of four fern species was assessed in relation to antifungal action. Extracts (fronds of dry period) at a concentration of 5 mg/mL inhibited the growth of the symbiont fungus *Leucoagaricus gongylophorus* less than 20% in tubes containing *Macrothelypteris torresiana* and *Dicksonia sellowiana* extracts, similar to the control. In the tubes containing *Niphidium crassifolium* extract, fungal growth inhibition was approximately 40%, similar to the control, whereas fungal growth was 100% inhibited by the *Parapolystichum effusum* extract (Fig. 2). On the other hand, none of the extracts (dry and rainy periods) inhibited symbiont fungal growth at a concentration of 100 µg/mL.To date, no studies on the biological potential of ferns in combating the symbiont fungus *L. gongylophorus* have been found in the literature, although Pereira *et al.* (2015), Banerjee & Pen (1980), Dolly *et al.* (2010) have reported the antifungal activity of fern extracts against other fungi, including *Curvularia luneta*, *Aspergillus niger*, *Helminthosporium oryzae*, *Candida albicans*, *Microsporum gypseum*, *Trichophyton mentagrophytes*, and *Trichophyton rubrum*. However, several angiosperm extracts showed antifungal potential against the symbiont fungus *Leucoagaricus gongylophorus*, such as *Handroanthus vellosoi* (Toledo) Mattos (yellow ipê), *Azadirachta indica* A.Juss. (neem), *Magonia pubescens* A.St.-Hil. (tingui), *Annona reticulata* L. (ox heart), *Amburana acreana* (Ducke) A.C.Sm. (Spanish oak) and *Sesamum indicum* L. (sesame) (Pagnocca *et al.* 1990; Souza *et al.* 2011).

Synthetic insecticides are often used to combat leafcutter ants, causing substantial environmental damage (Boulogne *et al.* 2021; Nickele *et al.* 2013). Thus, an environmentally-friendly strategy is necessary for more sustainable agriculture (Riaz *et al.* 2023). In the case of leafcutter ants, eliminating their mutualist fungi has shown to be very efficient in their combat. In this respect, the search for substances with antifungal activities in plant extracts is a promising strategy (Pagnocca *et al.* 1990; Rezende *et al*. 2007; Souza *et al.* 2011; Pereira 2012; Oliveira 2015). However, it is important to search for antifungal substances that are biosafe for the environment and human health, and that can replace the synthetic substances, many of which are persistent in water and soil, and toxic to different organisms that perform important ecosystem services (Duarte *et al.* 2022).

The low consumption or rejection of *Macrothelypteris torresiana*, *Dicksonia sellowiana* and *Niphidium crassifolium*, recorded by Mehltreter & Valenzuela (2012), may not be associated with their potential to inhibit *L. gongylophorus* growth. Ridley *et al.* (1996) described four situations of plant rejection by leafcutter ants: (1) when plants

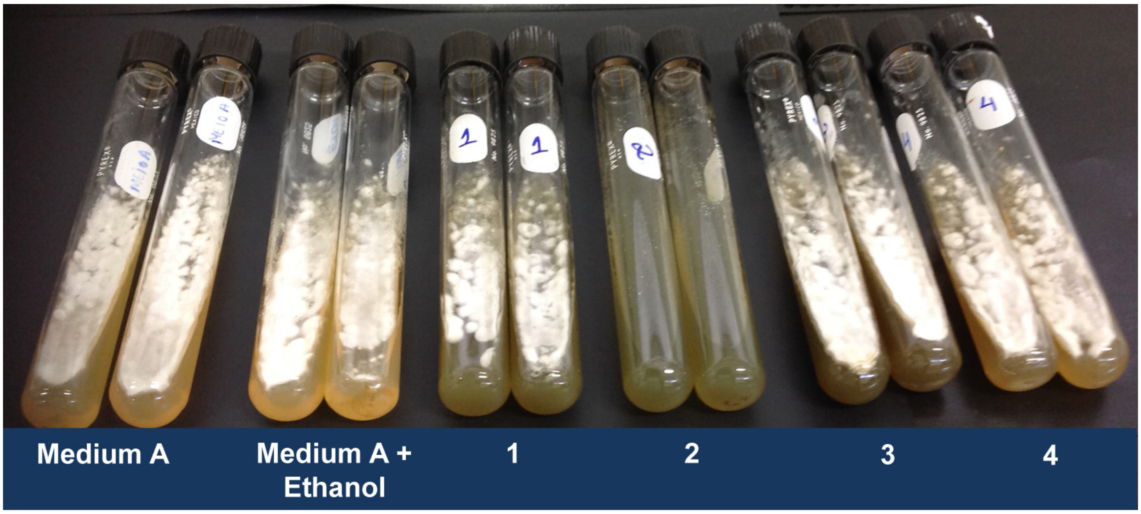

Figure 2 – Antifungal activity of hydroalcoholic extracts (5 mg/mL) of fern fronds (dry period) on the fungus *Leucoagaricus gongylophorus*. Controls: medium A and medium A + Ethanol. Extracts: 1 = *Niphidium crassifolium*; 2 = *Parapolystichum effusum*; 3 = *Dicksonia sellowiana*; 4= *Macrothelypteris torresiana*.

are inspected, but not cut, suggesting rejection of an unpleasant odor; (2) when cutting begins, but is not finished, indicating negative taste stimulus; (3) when leaves are cut but collected only once and then rejected, and (4) when recruitment ceases over time and then resumes, repeating this pattern continuously.

Occasional collection of *Parapolystichum effusum* fronds by ants (Mehltreter & Valenzuela 2012) is consistent with the findings of antifungal activity, given that initially accepted plant matter may be rejected between 10 and 16 hours after the onset of data collection, according to the fungal response to this substrate. As such, the colony may then avoid it for weeks or months (Ridley *et al.* 1996; Saverschek *et al*. 2010).

Chromatographic analyses for terpenoids and phenolic substances were conducted to characterize the chemical profile of these substances and their relationship with the activities displayed by the extracts. The chromatographic plates to assess the presence of terpenoids in the crude extracts of *Macrothelypteris torresiana* revealed 15 substances with retention factors in the rainy period and seven in the dry period; in *Dicksonia sellowiana*, nine substances for both periods; and in *Niphidium crassifolium*, nine and seven substances in the rainy and dry periods respectively. For the species *Parapolystichum effusum*, six substances were recorded in the rainy period and seven in the dry period (Chromatographic plates available on supplementary material <https://doi.org/10.6084/m9.figshare.22679422.v1>).

The dendogram constructed using the Sørensen similarity index, based on the presence/absence of retention factors (Rfs) in the TLC plate for terpenoids, showed the formation of two groups: (1) *Macrothelypteris torresiana* (dry and rainy period) with ≈30% similarity and (2) *Dicksonia sellowiana*, *Parapolystichum effusum* and *Niphidium crassifolium* with ≈35% similarity. *Parapolystichum effusum* (dry and rainy period) demonstrated ≈93% similarity. *Parapolystichum effusum* exhibited the greatest similarity in the presence of terpenoids between the dry and rainy periods (92.3%), followed by *Dicksonia sellowiana* (77.7%), *Niphidium crassifolium* (62.5%) and *Macrothelypteris torresiana* (27.2%) (Fig. 3).

The extracts of *Macrothelypteris torresiana* (rainy period), *Niphidium crassifolium* (dry period), and *Parapolystichum effusum* (rainy and dry periods) contained a substance with a retention factor equal to that of the beta-sitosterol standard. The *Dicksonia sellowiana* extract (rainy period) was the only one that was similar to the friedelinol standard. Ovesná *et al.* (2004) reported the antifungal activity of the terpenoid beta-sitosterol. Virtuoso *et al*. (2005) found activity in the same terpenoid against *Escherichia coli* and *Staphylococcus aureus.* According to Bernardes (2014), friedelinol

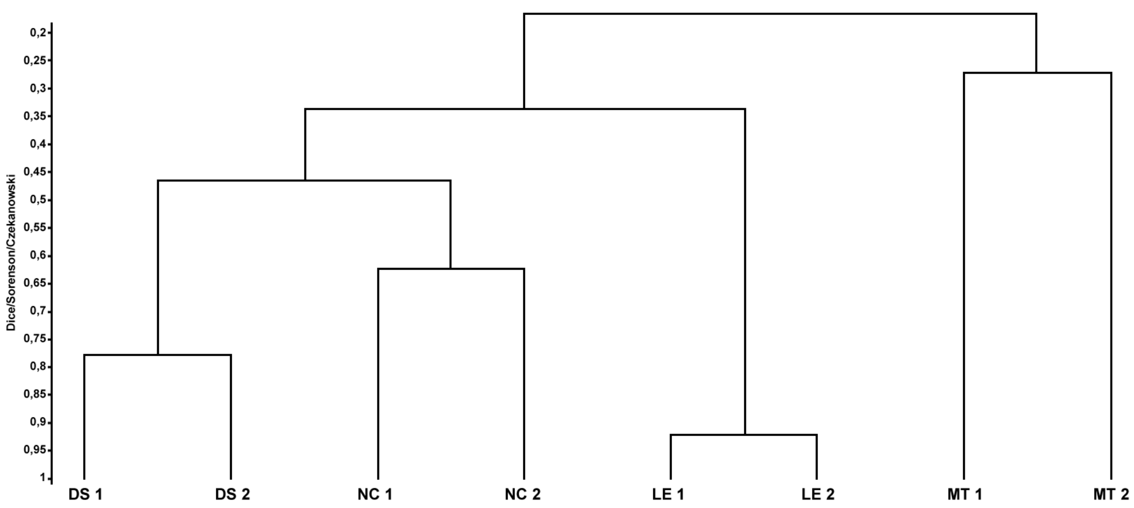

Figure 3 – Dendrogram based on the presence/absence of TLC retention factors (Rfs) for terpenoids. Rainy period: MT1 = *Macrothelypteris torresiana*; DS1 = *Dicksonia sellowiana*; NC1 = *Niphidium crassifolium*; LE1 = *Parapolystichum effusum*. Dry period: MT2 = *M. torresiana*; DS2 = *D. sellowiana*; NC2 = *N. crassifolium*; and LE2 = *P. effusum*. Cophenetic correlation = 0.9046.

showed inhibitory activity against the yeast *Candida albicans*. Friedelinol is a triterpene, compounds known to be precursors of phytosteroids, such as beta-sitosterol. In a phytochemical survey of ferns in Trinidad, Lynch *et al.* (1970) found no triterpenes in *Parapolystichum effusum*, only alkaloids.

Chromatographic plates for the presence of phenolic substances in crude extracts of *Macrothelypteris torresiana* revealed ten substances with retention factors in the rainy period and six in the dry period; in *Dicksonia sellowiana* six (rainy) and eight (dry); *Niphidium crassifolium* nine (rainy) and thirteen (dry); and *Parapolystichum effusum* two (rainy and dry) (Chromatographic plates available on supplementary material <https://doi.org/10.6084/m9.figshare.22679422.v1>).

The similarity dendogram for phenolic substances showed the formation of three groups: (1) *Parapolystichum effusum* (dry and rainy period) with ≈40% similarity; (2) *Dicksonia sellowiana* (dry and rainy period) with ≈30% similarity and (3) *Macrothelypteris torresiana* and *Niphidium crassifolium* with ≈35% similarity (Fig. 4).

Extracts of *Macrothelypteris torresiana* (rainy and dry periods), *Niphidium crassifolium* (rainy period) and *Parapolystichum effusum* (rainy period) contained a substance with Rf equal to that of the rutin standard. *Parapolystichum effusum* extract also contained a substance with Rf equal to that of the kaempferol standard.

Rutin and kaempferol are part of the flavonoid group denominated flavonols, known primarily for their antioxidant action. This action occurs due to the presence of hydroxyl in three rings (Cao *et al.* 1997). According to Han (2009), rutin also exhibits antifungal activity against *Candida albicans*. Kaempferol displays anti-inflammatory and anticarcinogenic activity against UV radiation and insect attraction (Cao *et al*. 1997; Ferreira *et al.* 2008).

Macrothelypteris torresiana shows the greatest variation (62.5%) for the presence of phenolic substances between the dry and rainy periods, followed by *Niphidium crassifolium* (45.4%), *Parapolystichum effusum* (40%), and *Dicksonia sellowiana* (28.5%).

Parapolystichum effusum (92.3%) and *Dicksonia sellowiana* (77.7%) showed the greatest terpenoid similarity between the dry and rainy periods, followed by *Niphidium crassifolium* (62.5%) and *Macrothelypteris torresiana* (27.2%),

The difference in chemical profile between the two periods may be due to the changes in resource supply such as light, temperature, water, and nutrients. According to Gobbo-Neto & Lopes (2007), several factors can cause potential changes in the chemical constitution of plants, seasonality being one of the main factors, since plants use their constituents to respond to stimuli provoked by the environment. A number of substances, such

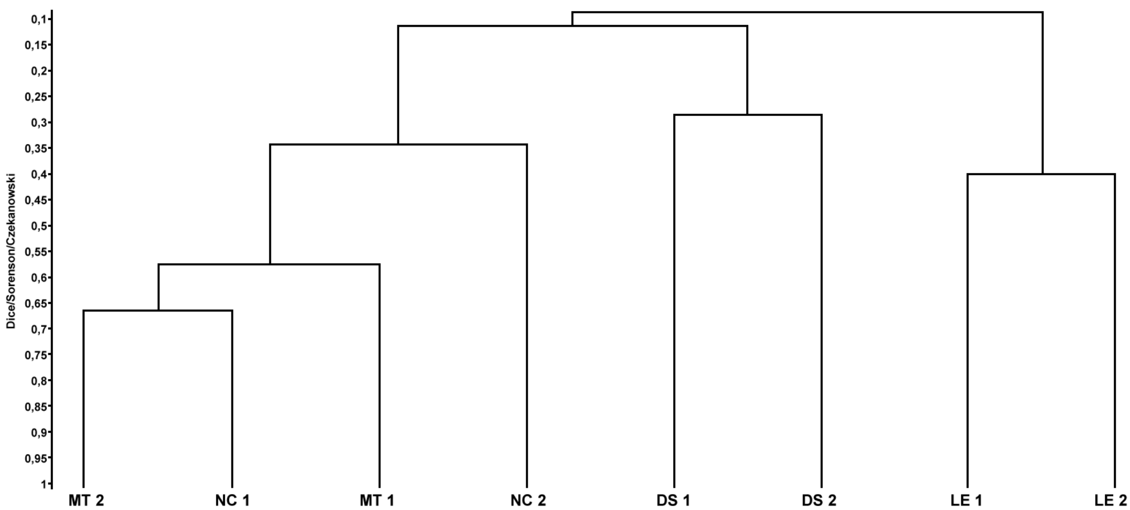

Figure 4 – Dendrogram based on the presence/absence of TLC retention factors (Rfs) for phenolic substances. Rainy period: MT1 = *Macrothelypteris torresiana*; DS1 = *Dicksonia sellowiana*; NC1 = *Niphidium crassifolium*; and LE1 = *Parapolystichum effusum*. Dry period: MT2 = *M. torresiana*; DS2 = *D. sellowiana*; NC2 = *N. crassifolium*; and LE2 = *P. effusum*. Cophenetic correlation = 0.8755.

as condensed tannins and monoterpenes, exhibit quantitative variations (Koricheva & Barton 2012). These authors report that the type and number of metabolites produced are related to plant appearance and how it attracts herbivores, that is, the hypothesis of evolution in the temporal change of metabolites. Also according to this hypothesis, quantitative defenses are found on apparent, long-lasting parts, such as mature leaves, while qualitative defenses appear on less apparent ephemeral regions, such as young leaves.

In conclusion, the results of the present study corroborate the ideia that insect-plant interactions and animal behavior in detecting chemical substances in plants may contribute to future studies of plant substances with antifungal action, especially ferns, a plant group frequently neglected. The crude extracts of *Parapolystichum effusum* and *Niphidium crassifolium* were the most promising in determining the minimum inhibitory concentration and molecular identification, aimed at helping in future formulations of antifungal products against the *Leucoagaricus gongylophorus,* mutualist fungus of leafcutter ants. Beta-sitosterol and rutin, substances known for their antifungal activity, were recorded for the two fern species. In this way, the ferns are promising plants in the search for environmental friendly substances for a sustainable agriculture.

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Data availability statement

In accordance with Open Science communication practices, the authors inform that supplementary data is available at <https://doi.org/10.6084/m9.figshare.22679422.v1>.

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