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Screening of native plants from central Argentina against the leaf-cutting ant *Acromyrmex lundi* (Guérin) and its symbiotic fungus

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ABSTRACT

Leaf-cutting ants are major agricultural and forestry pests in the New World. Attempts to control them have most frequently involved the use of chemical insecticides, with mixed results. Among alternative methods, botanical pesticides may provide a sustainable and efficient control of leaf-cutting ants. In the present study, we screened the activity of plant extracts derived from 89 species native to Argentina, against the leaf-cutting ant *Acromyrmex lundi* (Guérin) and its mutualistic fungus, *Leucoagaricus gongylophorus*, through a pick-up assay and bioautography, respectively. The pick-up assay revealed moderate to strong anti-foraging activity for just over 13.5% of the assayed species, including complete ant foraging inhibition for *Aristolochia argentina, Flourensia oolepis, Gaillardia megapotamica, Lantana grisebachii* and *Lithrea molleoides*. Most plant extracts were well tolerated by fungi, with only 12.3% of the species tested showing some degree of fungus growth inhibition. Among these, *A. argentina*, *F. oolepis* and *Pterocaulon alopecuroides* were the strongest inhibitors, whereas *Baccharis flabellata, Dalea elegans* and *Zanthoxylum coco* revealed a more moderate activity. Only *A. argentina* and *F. oolepis* extracts showed strong anti-foraging effects and affected fungus growth at the same time. Values of IC_{50} and MIC indicated that extracts inhibiting ant foraging at lower concentrations did not necessarily also inhibit fungus growth at lower doses. The active principle of *A. argentina*, on both ant foraging and fungal growth, was identified as argentilactone.

1. Introduction

Leaf-cutting ants in the genera *Acromyrmex* and *Atta* (Hymenoptera: Formicidae) are key neotropical herbivores, responsible for the removal of enormous quantities of plant biomass (Costa et al., 2008; Folgarait, 1998; Hölldobler and Wilson, 1990). Cut leaves are transported by the ants into the nests, to be used as compost substrate for cultivation of their symbiotic fungus, the basidiomycete *Leucoagaricus gongylophorus* (Singer) Möller (Schiott et al., 2008). The fungal symbiont, encoding a diversity of enzymes, drives plant biomass degradation and provides the ants with easily digested food in the form of specialized nutrient-rich hyphal tips, the gongylidia (Aylward et al., 2013).

Leaf-cutters forage on an impressively diverse array of plants throughout the year and are major agricultural and forestry pests in the New World (Zanetti et al., 2003), cutting up to 15% of the standing leaf crop and half of plant species in Central and South American forests (Wirth et al., 2003). In addition to leaves, the ants gather other plant structures such as flowers, fruits and seeds (Franzel and Farji-Brener, 2000).

Traditionally, attempts to control leaf-cutting ants have involved the use of chemical insecticides, with only relative success despite continuous applications (Cherret, 1986; Della Lucia et al., 2014; Schoereder et al., 2012). Although thousands of worker ants could be killed by insecticide applications, there is low probability of reaching the queen, a crucial step for colony collapse. The use of chemical insecticides is also strongly questioned because of the lack of specificity, potential bioaccumulation problems and contamination of ground water and agricultural products (Mugni et al., 2011; Wang et al., 2009). Consequently, alternative methods providing

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sustainable and efficient control of leaf-cutting ant colonies have been called for (Della Lucia et al., 2014).

An increasingly explored avenue for safe pest control is represented by botanical pesticides derived from plant extracts, with compounds that modulate leaf-cutting ant behavior being particularly promising (Della Lucia et al., 2014). A variety of plant extracts have shown negative effects on leaf-cutting ants and / or their symbiotic fungus (Santos et al., 2013). However, very few studies have simultaneously addressed the effects of plant extracts on ants and fungi, and they have generally dealt with a single plant extract. Thus, deleterious effects of *Ricinus communis* (Euphorbiaceae), *Simarouba versicolor* (Simaroubaceae) and *Spiranthera odoratissima* (Rutaceae) on the leaf-cutting ant *Atta sexdens* L. and its symbiotic fungus, have been recorded (Bigi et al., 2004; Peñaflor et al., 2009; Terezan et al., 2010) with other authors testing seed oil from three citrus species on a similar ant-fungus system (Fernandes et al., 2002).

Here, we simultaneously screen for the first time, the activity of a wide array of plant extracts against the leaf-cutting ant *Acromyrmex lundi* (Guérin) and its mutualistic fungus. One of the most frequent leaf-cutting ant species in Argentina, *A. lundi* is well known as a garden and agricultural pest (Caffarini et al., 2008; Caffarini et al., 2002). Its negative incidence has lately increased with the implementation of direct sowing for various crops such as wheat, soybean and corn, which favors ant population growth by avoiding the nest destruction typically associated to soil laboring (Ríos de Salusso, 2010).

We focused our study on the discovery of substances capable of inhibiting the foraging behavior of leaf-cutting ants and/or the growth of symbiotic fungi. Extracts inhibiting *A. lundi* foraging behavior could protect plants by preventing ant attack, while extracts with antifungal properties against *L. gongylophorus* could interfere with food production and thus hinder ant colony growth and survival. The first approach can lead to leaf-cutting ant control via plant protection and the second one via colony regulation.

Within this framework, extracts from 89 species belonging to 29 botanic families and growing native or naturalized in central Argentina, were assayed both in pick-up and antifungal tests to determine their activity against *A. lundi* foraging behavior and fungal growth, respectively. By concentrating mostly on native plant species we hoped to contribute to boost the value of natural vegetation. Also, while looking for promising ant control tools by screening extracts for anti-foraging and antifungal activity, we ask: do antifungal extracts tend to be rejected by foraging ants? If both activities coexist, are the more effective anti-foraging extracts also the stronger fungus inhibitors? Finally, we analyzed the active component of the most promising extract.

2. Materials and methods

2.1. Plant material

Plants were collected in the Chaco Serrano forest region (30°25′ to 31°59′S, 64°21′ to 65°00′W) within Córdoba Province, Argentina, from November 2009 to December 2011. Voucher specimens have been deposited in the "Marcelino Sayago" Herbarium of the School of Agricultural Science, Catholic University of Córdoba, and were botanically classified by the botanist Gustavo Ruiz. The plant material was air-dried at room temperature, crushed, extracted by 48 h maceration with ethanol (95%) and separated by filtration; the solvent was then vacuum evaporated to obtain syrupy extracts. The yield of each extract was calculated as percentage weight of air-dried plant material (see Supplementary Table 1 for plant taxonomic information and yield).

2.2. Artificial ant nest

The ant colony was kept in a plastic chamber (50 cm long × 30 cm wide × 25 cm high) (hereafter "nest") provided with 6 lateral holes and a tight cover lid. The colony was collected in the grounds of Catholic University of Cordoba. Thousands of workers, eggs and larvae, together with the queen ant and fungus garden were carefully moved to the nest chamber. Then, a foraging box (30 cm × 15 cm × 7 cm) and a garbage disposal box (50 cm × 30 cm × 12 cm) were each connected to the main chamber or nest by a transparent plastic tube (2.5 cm in diameter; 80 cm in length), using two opposite holes (Vieira-Neto et al., 2006). Two more holes were used to ventilate the colony through finely perforated stoppers and another one allowed connection to the foraging arena with control and treatment boxes used in the pick-up assay (see Section 2.3). The complete arrangement was maintained in a room conditioned at 26 °C, with 70% relative humidity and 12 h/12 h dark/light cycle. The colony was provided with 30–50 g/day leaves from various plant species (*Oxalis* sp., *Rosa* sp., *Ulmus* sp.) or corn flakes and water ad libitum. The garbage box was cleaned up every two days.

2.3. Pick-up bioassay

This assay was conducted on a specially designed foraging arena consisting in two parallel plastic boxes (50 cm × 30 cm × 12 cm) connected to the nest by plastic tubing (2.5 cm in diameter) in a "Y" design. For each extract test, ten 1 cm^2 rose (*Rosa* sp.) leaf pieces treated (20 μL) with extract solution (5 mg/mL, solvent acetone) at the rate of 100 μg extract/cm^2 were placed in one of the parallel boxes (treatment box) and 10 similar pieces treated (20 μL) only with solvent (acetone) were placed in the other box (control box). Once the leaf pieces were in position, the tubing was plugged into the nest through a lateral hole in order to let the ants onto the foraging arena. The insects immediately walked into the two boxes and, after an evaluation interval, started to pick up some of the offered substrate. The assay was carefully monitored until half of the total offered material was removed by the ants (Matthews, 1997), and the number of treatment and control pieces collected was recorded. An anti-foraging index (AFI) was calculated for each extract as $(C-T)/(C+T) \times 100$, where T and C indicate the number of treated and control leaf pieces, respectively, collected by the ants (Nathan, 2006). For this index, values may range from −100 (forage stimulant) to +100 (forage deterrent or anti-foraging), adapted from (Miles et al., 2005). We considered that AFI values between −70 and 70 indicated no or weak activity, while extracts with AFI >70 and <95 were considered moderate and AFI >95 were strong foraging inhibitors (Hassanali and Bentley, 1987). Given the high number of extracts tested and our interest in the detection of highly promising compounds for ant control, only those with strong anti-foraging effect (AFI = 100 in the initial test) were subjected to deeper analyses, with three repetitions (Matthews, 1997). The most active extracts or pure active principle were further assayed by triplicate at different concentrations (100 μg/cm^2, 50 μg/cm^2, 25 μg/cm^2, 12.5 μg/cm^2, 6.2 μg/cm^2, 3.1 μg/cm^2 and 1.5 μg/cm^2) and inhibitory concentration 50 (IC_{50}) values were calculated by probit analyses, using the AFI values.

2.4. Fungus

Six fragments of *A. lundi* fungus garden were collected and carefully freed of any ant workers and brood (Rodrigues et al., 2008), then placed into sterile Petri dishes containing a cotton wool piece imbibed with sterile distilled water. All plates were incubated at 25 °C in a cultivation chamber for 7–14 days in the dark (Rodrigues et al., 2008). Once the fungal mycelium was developed, pure

cultures were obtained by inoculating samples of this mycelium onto potato dextrose agar (PDA 2%, DIFCO®) plates. Morphological characteristics and the presence of gongylidia were used as indicators of the isolated fungus being *L. gongylophorus* (Schiott et al., 2008).

2.5. Bioautography and antifungal assay

In order to screen the antifungal activity of the extracts, direct bioautography was carried out on TLC plates (Carpinella et al., 2010) as follows. Each extract, dissolved in acetone, was applied in a TLC plate at a dose of 500 μg/spot and, after the solvent was evaporated, a fungus inoculum suspended in glucose-mineral salts medium (Carpinella et al., 2005), was sprayed directly onto the plate. The inoculum was prepared by suspending the fungi of four to five-day-old cultures, in glucose-mineral salts medium till reaching an absorbance of 0.022 at 530 nm. Glucose-mineral salts medium was prepared by dissolving 7 g KH_2PO_4, 3 g $Na_2HPO_4{\cdot}2H_2O$, 4 g KNO_3, 1 g $MgSO_4{\cdot}7H_2O$, 1 g NaCl in 1 l of tap water. After sterilization of this solution it was mixed with a freshly prepared solution of glucose 30% previously filtrated by a 0.45 μm membrane. A plate treated only with acetone (control) was also sprayed with the fungal inoculum. The plates were then incubated in a chamber at 25 °C and in darkness. At the end of the incubation period (48 h), the extension (in mm) of a clearly visible growth inhibition zone was measured (Carpinella et al., 2003). All treatments were run in triplicate. For mean inhibition areas of 0 mm, 5 mm, 10 mm and 15 mm, the extract antifungal activity was expressed as inactive (−), weak (+), moderate (++) and strong (+++), respectively (see Table 1).

For extracts showing strong antifungal activity or pure active principle, the Minimum Inhibitory Concentration (MIC) was assessed via broth microdilution, carried out in a 96-well sterilized microplate, with synthetic fungicide carbendazin being used as positive control. The inoculum was prepared with four to five-day-old *L. gongylophorus* colonies and transferred to sterile glucose-mineral salts medium (5 mL) to reach a suspension with optical density of 0.022 at 530 nm. Extracts were dissolved in acetone and incorporated into each well containing the fungi suspension. The extract final concentration ranged from 0.01 to 500 μg/mL. The final concentration of acetone in each well was 2%. Wells containing the inoculum with or without acetone addition (2%) were simultaneously run as controls. These experiments were run in triplicate. The percentage of fungus growth in each well with regards to controls (considered as 100% growth) was assessed by observation with an inverted light microscope for each extract concentration. All measurements were done by the same operator. MICs were determined as the lowest concentration of each extract that completely inhibited fungus growth.

2.6. Bioguided isolation of antiforaging and antifungal active principle

Air-dried aerial parts of *A. argentina* (430 g), the plant species that showed the highest activity in the screening here performed, were extracted with ethanol for 24 h at room temperature. After solvent removal (reduced pressure), the extract obtained (11.2 g, 2.6% yield) showed antiforaging activity AFI = 100% at 100 μg/cm^2 against *A. lundi*, and fungal inhibition (+++) at 500 μg/spot. The extract (1.8 g) was fractionated by silica gel column chromatography and 11 fractions were eluted with a gradient of hexane/Et_2O and MeOH. The active fractions 3–4 (AFI = 100% at 50 μg/cm^2), which were eluted with hexane/Et_2O 70:30, were then rechromatographed on silica gel with a gradient of hexane/Et_2O from 100% hexane to 100% Et_2O, obtaining 8 fractions of which fraction 3 was active against both *A. lundi* and the symbiontic fungus (AFI = 100%, fungal inhibition (+++)). From this fraction, an oily

Table 1
Effects of extracts from native plants from Central Argentina on *Acromyrmex lundi* foraging behavior (AFI) and growth of its mutualistic fungus (AF).

Plant species	AFI	AF
Aristolochia argentina Griseb	100	+++
Flourensia oolepis S.F. Blake	100	+
Gaillardia megapotamica (Spreng.) Baker var. *megapotamica*	100	–
Lantana grisebachii Stuck. *ex* Seckt var. *grisebachii*	100	–
Lithrea molleoides (Vell.) Engl.	100	–
Cantinoa mutabilis (Rich.) Harley & J.F.B. Pastore	88.2	–
Podronea ricasoliana (Tanfani) Sprague	77	–
Achyrocline satureioides (Lam.) DC.	75	–
Aloysia citrodora Palau	75	–
Dipsacus fullonum L.	75	–
Lippia turbinata Griseb.	75	–
Monnina dictyocarpa Griseb.	75	–
Gomphrena pulchella Mart.	69	–
Aloysia gratissima (Gill.& Hook.) Tronc.	66	–
Acacia aroma Gillies *ex* Hook. &Arn	60	–
Cotoneaster glaucophylla Franch.	60	–
Jodina rhombifolia (Hook. & Arn.) Reissek	60	–
Ruprechtia apetala Wedd.	60	–
Zexmenia buphtalmiflora (Lorentz) Ariza	60	–
Pavonia aurigloba Krapov. & Cristóbal	55.5	–
Acalypha communis Müll. Arg.	50	–
Astragalus distinens Macloskie	50	–
Baccharis artemisioides Hook. & Arn.	50	–
Bidens pilosa L.	50	–
Solanum palinacanthum Dunal	50	–
Vernonanthura nudiflora (Less.) H. Rob.	50	–
Baccharis sessiliflora Vahl	46.7	–
Verbesina encelioides (Cav.) Benth. & Hook. *f. ex* A. Gray	45	–
Acacia atramentaria Benth.	40	–
Melissa oficinalis L.	40	–
Minthostachys verticillata (Griseb.) Epling	37.5	–
Ligaria cuneifolia (Ruiz & Pav.) Tiegh.	35	–
Ambrosia elatior L.	33.3	–
Anemia tomentosa (Savigny) Sw.	33.3	–
Angelphytum aspilioides (Griseb.) H. Rob.	33.3	–
Baccharis salicifolia (Ruiz & Pav.) Pers.	33.3	–
Lepechinia floribunda (Benth.) Epling	33.3	–
Solanum argentinum Bitter & Lillo	33.3	–
Ophryosporus charrua (Griseb.) Hieron.	33	–
Lorentzianthus viscidus (Hook. & Arn.) R.M. King & H. Rob.	29	–
Grindelia pulchella Dunal	25	+
Cynoglosum amabile Stapf & J.R. Drumm.	25	–
Dolichandra cynanchoides Cham.	25	–
Sida rhombifolia L.	25	–
Senecio vira-vira Hieron.	23	–
Amphilophium carolinae (Lindl.) L. G. Lohmann	16.7	–
Kageneckia lanceolata Ruiz & Pav.	16.7	–
Artemisia annua L.	14	–
Porlieria microphylla (Baill.) Descole, OîDonell & Lourteig	6.7	–
Melinis repens (Willd.) Zizka	6.6	–
Baccharis flabellata Hook. & Arn.	0	++
Dalea elegans Gillies *ex* Hook. & Arn. var. *elegans*	0	++
Baccharis coridifolia DC.	0	+
Baccharis linearifolia (Lam.) Pers.	0	+
Trichocline reptans (Wedd.) Hieron.	0	+
Argemone subfusiformis G. B. Ownbey	0	–
Capparis atamisquea Kuntze	0	–
Dysphania ambrosioides (L.) Mosyakin & Clemants	0	–
Croton lachnostachyus Baill.	0	–
Eryngium horridum Malme	0	–
Acanthostyles buniifolius (Hook. & Arn.) R.M. King & H. Rob.	0	–
Chromolaena hookeriana (Griseb.) R.M. King & H. Rob.	0	–
Marrubium vulgare L.	0	–
Microliabum candidum (Griseb.) H. Rob.	0	–
Prosopis alba Griseb. var. alba	0	–
Salvia cuspidata subsp. *gilliesii* J.R.I. Wood & Harley	0	–
Senecio madagascariensis Poir.	0	–
Sphaeralcea cordobensis Krapov.	0	–
Tagetes minuta L.	0	–
Viguiera tucumanensis (Hook. & Arn.) Griseb.	0	–
Dolichandra unguis-cati (L.) L.G. Lohmann	−5.3	–
Flourensia campestris Griseb.	−11	–
Mandevilla pentlandiana (A. DC.) Woodson	−11	–
Thalictrum decipiens Boivin	−11	–
Achyrocline tomentosa Rusby	−20	+

Table 1 (*Continued*)

Plant species	AFI	AF
Vernonia mollissima Hook. & Arn.	−20	–
Zanthoxylum coco Hook.f. & Arn.	−25	++
Solanum sisymbriifolium Lam.	−25	–
Thelesperma megapotanicum (Spreng.) Kuntze	−25	–
Elaphoglossum lorentzii (Hieron.) H. Christ	−33	–
Lepechinia meyenii (Walp.) Epling	−33	–
Pyrostegia venusta (Ker Gawl.) Miers	−41	–
Pterocaulon alopecuroides (Lam.) DC.	−45	+++
Artemisia verlotiorum Lamotte	−45	–
Araujia brachystephana Griseb.	−50	–
Condalia microphylla Cav.	−50	–
Cortaderia rudiuscula Stapf	−75	–
Senna aphylla (Cav.) H.S. Irwin & Barneby	−76	–
Jarava ichu Ruiz & Pav.	−100	–

AFI: anti-foraging index (see text). AF: anti-fungal activity; (−) 0 mm, (+) 5 mm, (++) 10 mm, (+++) 15 mm area of fungus growth inhibition.

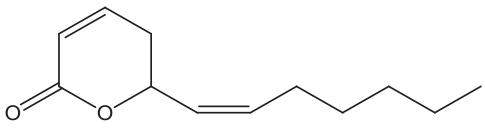
material was isolated (81 mg) and identified as argentilactone (Fig. 1) by ^{1}H NMR, ^{13}C NMR (Bruker AVANCE II 400 spectrometer, Bruker Corporation, Ettlingen, Germany, chemical shifts in in parts per million, relative to internal tetramethylsilane for $\delta = 0.00$) and MS techniques (electron impact mass spectra obtained at 70 eV by GC–MS on a Hewlett-Packard 5970 Series mass spectrometer interfaced with a Hewlett-Packard 5890 gas chromatograph fitted with a HP-5MS, column 15 m × 0.25 mm i.d., temperature from 100 to 280 °C, 10 °C/min).

Argentilactone: ^{1}H NMR(400 MHz, $CDCl_3$): δ 6.89 (ddd, 1H, J = 11.2, 4.2, 2.0 Hz), 6.04 (ddd, 1H, J = 11.2, 2.2, 1.6 Hz), 5.67 (m, 1H), 5.56 (m, 1H), 5.22 (ddd, 1H, J = 9.6, 8.8, 4.6 Hz), 2.47–2.31 (m, 2H), 2.17–2.02 (m, 2H), 1.42–1.26 (m, 6H), 0.89 (t, 3H, J = 7.2 Hz). ^{13}C NMR (100 MHz, $CDCl_3$): δ 164.2, 144.7, 135.7, 126.4, 121.6, 73.9, 31.3, 29.9, 29.1, 27.8, 22.5, 13.9. MS, m/z (rel. int.): 194 $[M]^+$ (2), 152 (1), 137 (2), 119 (2), 110 (4), 109(4), 97 (14), 86 (8), 68 (100), 55 (22). The spectral data were identical to previously published data for argentilactona (de Fatima et al., 2004; Priestap et al., 1977).

3. Results and discussion

3.1. Anti-foraging activity

The effects of the 89 plant extracts on the foraging behavior of *A. lundi*, expressed as the anti-foraging index AFI, are presented in Table 1. The pick-up assay revealed moderate or strong anti-foraging activity for just 13.5% of the assayed species (Table 1). Instead, over two thirds of the extracts did not show relevant effects on ant foraging activity, and even a small fraction of plant extracts were attractive to foraging ants (Table 1). The low activity level of a large proportion of the plant extracts here tested, agrees with the broad array of plants that are usually collected by leaf-cutting ants (Rockwood, 1976). Nonetheless, some extracts hindered ant foraging and, most interestingly from a pest management point of view, those of *Aristolochia argentina*, *Flourensia oolepis*, *Gaillardia megapotamica*, *Lantana grisebachii* and *Lithrea molleoides* were completely rejected by the ants. None of these plant species have been



Argentilactone

Fig. 1. Molecular structure of the antiforaging and antifungal active principle of *Aristolochia argentina*.

Table 2
Mean inhibitory concentration for *A. lundi* foraging activity (IC_{50}) and minimum inhibitory concentration against the symbiotic fungus (MIC) for the most active plant extracts in each category.

Plant/compound	IC_{50} in μg/cm^2 (95% CI)	MIC (μg/mL)
Aristolochia argentina	9.74 (8.25–11.51)	1.95
Lantana grisebachii	12.53 (11.03–14.25)	>500
Lithrea molleoides	21.32 (18.62–24.40)	>500
Flourensia oolepis	41.11 (22.73–54.35)	7.80
Gaillardia megapotamica	61.96 (39.42–87.00)	>500
Pterocaulon alopecuroides	>150	7.80
Argentilactona	5.66 (2.55–10.53)	0.90
Carbendazin[a]		0.12

[a] Positive fungicide control. CI: confidence limit following probit analysis at $p < 0.05$.

previously reported as regulators of ant cutting behavior (Santos et al., 2013).

Some of the plant species here assayed, such as *Aloysia citrodora*, *A. gratisima*, *G. megapotamica*, *Lippia turbinata*, *Melissa officinalis*, *Minthostachys verticillata* and *Tagetes minuta* are known to produce terpenes which might be toxic to insects (Palacios et al., 2009), however only one of those species (*G. megapotamica*) showed strong antiforaging effect in our tests. Besides restraining leaf cutting and gathering, we observed that *G. megapotamica* extracts induced changes in the foraging pattern of *A. lundi*, including lethargy and arrestant effects on ant behavior. Arrestant behavior was reportedly induced by β-caryophyllene on the leaf-cutting ant *A. sexdens rubropilosa* (North et al., 2000), suggesting that the observed effects might be attributed to the presence of caryophyllene, a component of *G. megapotamica* essential oil (Adams et al., 2008).

Although leaf-cutting ants are generalist foragers that cut and collect leaves from a wide variety of plant species, they can also be highly selective, preferring or rejecting particular species (Franzel and Farji-Brener, 2000). In a previous study (Del Corral et al., 2014; Palacios et al., 2007) we assayed antifeedant activity for all of the extracts here studied, against the specialized herbivorous beetle *Epilachna paenulata*. Notably, none of the best antifeedants for *E. paenulata* showed high anti-foraging activity on *A. lundi*, suggesting that ants may not select plants following the same criteria of chewing herbivore insects. However, there is also a great variability among chewing insects in their responses to plant compounds (Castillo et al., 2013; Defagó et al., 2009). On the other hand, some of the extracts appeared to be attractive to foraging ants. Although the increase in foraging activity was mostly weak and was not the objective of our study, attractants could be used as baiting in association with toxic compounds or capture tools, in ant management programs (Della Lucia et al., 2014).

The plant extracts with strong inhibitory effect on ant foraging, in terms of AFI values (Table 2), were effective at concentrations comparable to the effective doses of pure compounds (Defagó et al., 2006; Diaz Napal et al., 2009; Marsaro et al., 2004), indicating that they are likely to contain strong active principles.

3.2. Antifungal activity

Results from the antifungal tests are also shown in Table 1. Most plant extracts had virtually no effect on fungal growth, with only 6% of the species tested inducing moderate or strong fungus growth inhibition (Table 1). Among these, *A. argentina* and *Pterocaulon alopecuroides* were the strongest inhibitors. Although the minimum inhibitory concentrations of these extracts were higher than the positive control carbendazin (Table 2), their activity against the symbiotic fungus appears highly relevant considering that they are crude extracts. Among the extracts that negatively affected the ant symbiotic fungus, those of *A. argentina*, *D. elegans*, *F. oolepis*, *T. reptans* and *Z. coco* have also been reported as growth inhibitors on

Fusarium verticillioides (Carpinella et al., 2010), suggesting a wider antifungal activity.

3.3. *Active principle of* Aristolochia argentina

Since the extract of *A. argentina* showed the highest inhibitory activity on both ant foraging and fungal growth, we searched for the active principles involved in each activity. Through a bioguided process, a lactone (81 mg, 4.5% yield) (Fig. 1) was isolated as the most active antiforaging and antifungal principle and identified as argentilactone (de Fatima et al., 2004).

Argentilactone was previously found in the essential oil and extracts of different parts of *A. argentina* (Priestap et al., 1977), *Annona haematantha* (Waechter et al., 1997), *Hyptis ovalifolia* (Oliveira et al., 2004) and *Chorisia crispiflora* (Saeed et al., 2001). Various biological activities such as antifungal (Oliveira et al., 2004), anti-leishmanial (de Carvalho and Ferreira, 2001), trypanocidal (de Fátima et al., 2006) and cytotoxicity (de Fatima et al., 2004) have been reported for this compound, but its anti-foraging activity was not previously known. The dosage necessary for 50% foraging inhibition (IC_{50}) and the MIC against *L. gongylophorus* was reduced by about half when using argentilactone, in comparison with the full *A. argentina* extract (Table 2).

3.4. *Antiforaging and antifungal activity link*

Only *A. argentina* and *F. oolepis* extracts showed strong antiforaging effects while also inhibiting fungus growth. Therefore, comparison of antiforaging and antifungal activities through values of IC_{50} and MIC from highly active extracts in each category showed that extracts inhibiting ant foraging at lower concentrations did not necessarily inhibit fungus growth at lower concentrations as well (Table 2). The active principle of *A. argentina*, argentilactone, was highly effective in inhibiting the foraging ant behavior as well as symbiotic fungus growth, indicating that the ants would be able to detect a priori compounds that interfere with the normal development of the symbiont. Very few species have evolved specialized physiological or ethological adaptations allowing them to deal with aristolochic acid (Pinto et al., 2011), which is a typical chemical feature of *Aristolochia* species (Jacobson, 1982) however according our finding they are not seem to be involved in ant negative response to this plant extract.

Notably, in addition to *A. argentina* showing the lowest values for both IC_{50} and MIC (Table 2), such values were four times lower in comparison with *F. oolepis,* suggesting a link between antifungal and antiforaging activities. An antifungal-antiforaging link suggests a defensive strategy on the part of the ants, rejecting compounds that are deleterious to their symbiotic fungus, which could be exploited for plant protection against these pests. However other extracts with relatively low IC_{50} did not show antifungal activity, suggesting that the negative effect on ant foraging behavior could also be driven by mechanisms other than recognition of fungal inhibitors. On the other hand, when extracts with relatively low MIC and thus strong antifungal activity were weakly or not rejected, foraging ants might learn to reject chemicals injurious to the fungus (Ridley et al., 1996).

In summary, we have shown extracts from *A. argentina* and other native plant species from central Argentina to represent promissory tools for ant management, by inhibiting ant foraging and/or hindering growth of the symbiotic fungus. Our study hints at a naturally occurring link between antifungal and antiforaging activities, opening up interesting possibilities for plant protection through this dual mechanism converging in some plant extracts. Our results also highlight the importance of simultaneously addressing various types of activity in plant extracts, such as modulation of ant behavior and fungus growth inhibition, in order to propose natural materials for multiple-target control programs of leaf-cutting ant pests.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.indcrop.2015.07.001

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