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Chapter 9

Plant–Derived Products for Leaf–Cutting Ants Control

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1. Introduction

1.1. Leaf–cutting

Leaf-cutting ants of the genera *Atta* sp. Fabricius (Hymenoptera: Formicidae) and *Acromyrmex* sp. Mayr (Hymenoptera: Formicidae) are among the best known species of the family Formicidae in the New World, mainly due to their behaviour of cutting live plants to grow the symbiotic fungus *Leucoagaricus gongylophorus* (Möller) Singer (Agaricales: Agaricaceae) [1] (Figure 1). This interaction, which emerged more than 50 million years ago [2] has evolved to such a complex level that the ants and fungi cannot survive separately; they live in symbiosis. The fungus supplies the ants with nutrients obtained from metabolising plant materials that can be easily assimilated. In exchange, its environment is highly protected by the ants, which remove contaminants and secrete antibiotics from their metapleural glands [3, 4].

The symbiotic fungus, which exhibits high carbohydrate and protein content but low content, constitutes the main food source for leaf-cutting ant colonies [5] and is the single nutrient source for the queen, larvae, and temporary alate castes. Only 9% of the energy requirements of adult workers, which ingest plant sap when handling plant fragments, are obtained from the fungus [6]. Moreover, the symbiotic fungus produces large amounts of enzymes, which are ingested by the ants and are returned to the fungal garden through faecal liquid to facilitate the digestion of plant tissue [7, 8].

Leaf-cutting ants are considered the main agricultural and forest pest in countries such as Brazil, as they attack plants at any stage of their development, cutting their leaves, flowers, buds, and branches, which are then transported to the interior of their underground nest [9]. A colony of *Atta laevigata* (F. Smith) (Hymenoptera: Formicidae) can cut approximately 5 kg

Figure 1. *Atta sexdens* and its fungal garden: mycelial care. Source: Arnhold, 2012.

of plant material/day [10]. Thus, these ants cause direct losses, such as the death of seedlings and reduction of tree growth. Indirect losses also occur as a result of the decreased resistance of trees to other insects and pathogenic agents [11].

Leaf-cutting ant control has been performed almost exclusively through the application of conventional insecticides, including cyfluthrin (pyrethroid), imidacloprid (neonicotinoid), furathiocarb (carbamate), sulfluramid (fluoroaliphatic sulfonamide), and fipronil (phenyl pyrazole) [12]. Due to the problems these products may cause to the environment and humans, their use has been restricted by governments and forest product certification bodies, which have demanded and encouraged the development of alternative control strategies to these insecticides, such as the use of plant-derived products, entomopathogenic fungi, and pheromones [13].

Plant-derived products can be used to control ant populations through several mechanisms. Some of these substances can act directly against the ant, leading to its death, such as citrus seed oils obtained from *Citrus sinensis* (L.) Osbeck, *Citrus limon* (L.) Burm. f. or *Citrus reticulata* Blanco (Rutaceae) [14] and extracts from the castorbean (*Ricinus communis* L.) (Euphorbiaceae) [15], timbo ((*Ateleia glazioviana* Baill.) (Leguminosae) [16] and eucalyptus ((*Eucalyptus*

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urophylla S.T. Blake) (Myrtaceae) [17]. Certain plant-derived substances can promote aggressive behaviour of ants towards their sisters, as reported for β-eudesmol extracted from eucalyptus leaves. [18, 19, 20] This sesquiterpene is able to modify the chemical composition of the worker's cuticle, impairing nest recognition, which triggers warning and aggressive behaviours among ants [20]. Plant extracts can also be toxic to the symbiotic fungus (*L. gongylophorus*), which represents an interesting target for new products for ant control. Such effects can be observed for extracts of *R. communis*, *Helietta puberula* R.E.Fr. (Rutaceae), *Simarouba versicolor* St. Hill (Simaroubaceae), and *Canavalia ensiformis* (L.) DC. (Fabaceae) [15, 21-22 ,23].

2. Leaf–cutting ants of the genera *Atta* and *Acromyrmex* and their symbiotic fungus, *Leucoagaricus gongylophorus*

Leaf-cutting ants comprise over 12,000 species and are considered social insects because they participate in parental care, reproductive castes, overlapping generations, and a division of labour [24]. These insects live in permanent colonies and are holometabolous. Taxonomically, they belong to the order Hymenoptera, family Formicidae, subfamily Myrmecinae, and tribe Attini [25]. Leaf-cutting ants belong to the genera *Atta* Fabricius and *Acromyrmex* Mayr as well as the basal genera *Apterostigma* Mayr, *Kalathomyrmex* Klingeberg and Brandão, *Mycetagroicus* Brandão and Mayhé-Nunes, *Mycetarotes* Emery, *Mycetaphylax* Fr. Smith, *Paramycetophylax* Kusnezov, *Sericomyrmex* Mayr, *Trachymyrmex* Forel, *Pseudoatta* Gallardo, and *Attaichnus* Lazã. [21] The Attini tribe is found only in the Neotropical region and is distributed between southern South America and the southern United States [26].

The leaf-cutting ants of the genera *Atta* and *Acromyrmex* (known as *saúvas* and *quenquéns* in Brazil, respectively) build nests composed of hundreds of underground chambers that are connected to each other and to the surface through galleries. The exterior of the nest usually exhibits a loose soil mound originating from the chambers formed by the nest [27]. Holes can be found in the loose soil mound or outside of it. According to Mariconi (1970), a large anthill of approximately 200 m^2 may contain an estimated population of six million ants [28].

Leaf-cutting ants cultivate the symbiotic fungus *L. gongylophorus*, which is used as a source of food and of auxiliary enzymes that function in the ants digestive process [29]. After the origination of the ant-fungus symbiosis, a subsequent evolutionary step involved the acquisition of staphylae-rich monophyletic cultivars by the highly derived Attini (Figure 2), particularly the *Atta* and *Acromyrmex* species, which collect only fresh vegetation as the fungal substrate. The conversion of vegetation into substrate requires several different operations and specialisations. The plants are cut by workers who have a head width of 1.6 mm or greater (other tasks, such as defence, involve larger workers), while care of the fungus requires very small workers, and intermediary steps in the garden are performed by medium-sized workers. Among ant species, this great evolutionary change seems to have placed the derived Attini on the path of producing larger and larger colonies due to the increased differences between the

sizes of reproductive females and workers. Relatively larger reproductive ants show an increased ovariole count, enabling rapid colony growth [30], which, due to the increased size variation among nestmate workers, are able to execute all activities involved in fungal cultivation.

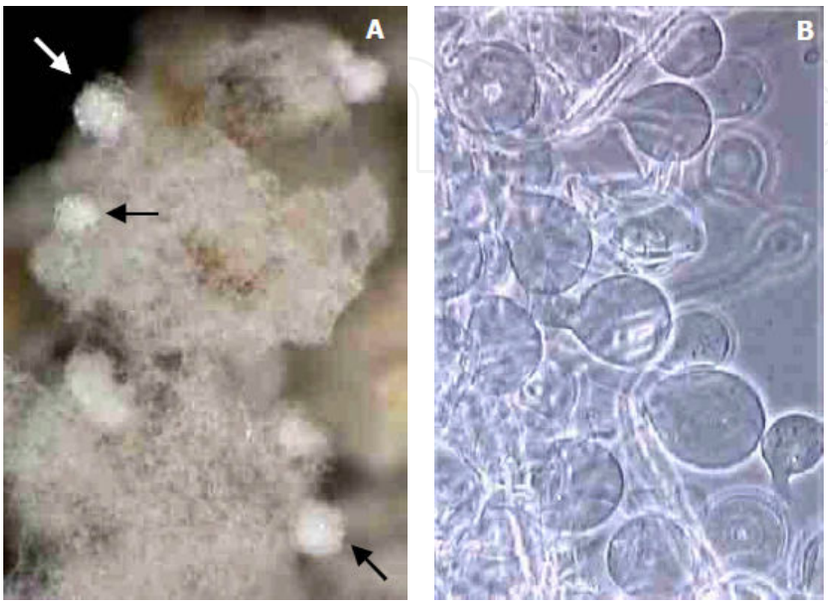

Figure 2. Microscopic images of *Leucoagaricus gongylophorus*, the symbiotic fungus of *Atta sexdens*. (A) Staphylae (arrows) contrasting with the hyphae (50x magnification). (B) Staphylae viewed under an optical microscope: each sphere corresponds to a gongylidia (1,000x magnification). Source: Schneider and Odair, 2003 [78].

Leaf-cutting ants from the genera *Atta* and *Acromyrmex* are the only ants within the group Attini that exhibit polyandry [31]. The development of this behaviour in the Attini coincided with the practice of cutting the leaves and live parts of plants [30]. The use of leaves, which are a widely available resource, would have allowed the leaf-cutting ants to achieve large colonies, leading to the appearance of long-lived queens and more complex forms of social organisation. Polyandry could favour the development of disease resistance as it increases the genetic diversity of the colony [32].

The consecutive matings between *Atta* and *Acromyrmex* species occur at the regional level throughout Brazil. In the southeast region, the nuptial flight of *Atta* species occurs between October and December; during this period, adult nests (approximately 38 months old) produce alate and fertile ants, and during the nuptial flight, referred to as swarming, the males fertilise the females [33]When leaving the nest for the nuptial flight, the virgin queen carries a small fragment of mycelium in her infrabuccal pocket to start a new fungus garden in a new nest [28]After mating, the reproductive females walk on the soil surface, remove their wings and search for a site to begin the excavation of the new nest. [34] In general, 48 hours after the excavation of the initial chamber [28], the future queen regurgitates the fungal fragment from her infrabuccal pocket. When starting the new colony, this small portion of the fungus is sterilised and fertilised with faecal droplets. Five to six days after the beginning of nest

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excavations, the queen begins oviposition [35]. The queen in a new colony lays both reproductive and trophic eggs, which are used as a food source for both the queens and their first offspring [36]. Early larvae originating from the reproductive eggs emerge 24 to 25 days after soil excavation by the queen. Ant larvae have been described as the digestive caste of the colony [25] and can actively contribute to the integration of the colony not only as a source of new adults but also by providing nutrients and enzymes for the workers, thus ensuring that the larvae will be cared for, which is essential for their survival. At 62 to 66 days after the nuptial flight, the first adults emerge, which feed on sap or obtain liquid food through trophallaxis or regurgitation [35]. On average, 87 days pass between the initial excavation and the appearance of the first opening created by workers. The second opening is only built 14 months after the first one. Additional openings appear quickly; on average, after 82 days, eight openings are present. The tenth opening is created approximately 20 months after the colony is fertilised. When the nest reaches three years of age (±38 months), it reaches adulthood and produces the first nuptial flight [37].

The use of plants as a substrate for the growth of the symbiotic fungus has led these ants to become the main pest on Brazilian forest plantations, despite playing a very important ecological role [38,39,40]. Leaf-cutting ants remove and modify the soil during the nest construction process, promoting changes in the chemical-physical proprieties of the soil and nutrient cycling that are favourable to plant growth [34]. However, their position as a key pest is generally cited more often due to the large quantities of vegetation these ants collect for growing the symbiotic fungus.

Leaf-cutting ants show a preference for certain plant species, which are consistently defoliated, while other species are not attacked, despite being abundant and located close to their nests. Some ant species attack only dicotyledonous plants, others target monocotyledons, and some ants collect both plant types. The preference for particular plant species may be related to the nutritional demands of the symbiotic fungus *L. gongylophorus* grown by these ants [41]. Ants can attack plants at any stage of their development, cutting their leaves, flowers, buds, and thin branches, which are then transported to the interior of their underground nests [42,43].

The complete defoliation of one-month-old *Pinus taeda* L. causes 25% mortality of these plants. For the plants that survive, defoliation affects their diameter more than their height [44]. Previous studies have demonstrated that there is a significant reduction in the development of *P. taeda* during the first 24 months of life when attacked by leaf-cutting ants [45]. Total defoliation reduces the growth of *Eucalyptus grandis* W. Hill ex Maiden in both diameter and height as well as the profit of the producer at the end of the rotation, even if the defoliation occurs only once during the beginning of the planting. The decreases in production and profits are enhanced according to the frequency of defoliation, which can make the maintenance of totally defoliated areas economically unviable. The damage caused by ants is more harmful to the plants in their first three years. A single total defoliation event delays the growth of the plant [46] while two or three consecutive defoliation events may lead to plant death [47,48].

Due to the damage they cause, control of these pests is essential to avoid large losses in agricultural and forest crops [33]

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3. Plants toxic to leaf–cutting ants and their symbiotic fungi

Plants exhibit several mechanisms to prevent herbivory, including producing alcohols, aldehydes, esters, phenols, and hydrocarbons, among other substances, which can be classified as secondary metabolites and can be toxic to leaf-cutting ants and/or their symbiotic fungi. As a result, many studies have been conducted in this field using different plant families, as listed in Table 1; some of these studies are described in more detail below.

Plant species	Family	Identified substance	Reference
Hymenaea courbaril	Fabaceae	Caryophyllene epoxide	Hubbel et al., 1983[80]
Sesamum indicum	Pedaliaceae	Sesamin and/or sesamolin	Pagnocca et al., 1990[49]; Pagnocca et al; 1996[50]; Ribeiro et al., 1998[51]; Morini et al., 2005[52]; Peres Filho and Dorval, 2003[53]
Canavalia ensiformes	Fabaceae	Fatty acids; canavalin, canatoxin	Monteiro et al., 1998[55]; Hebling et al., 2000[81]
Pilocarpus grandiflorus	Rutaceae	Vanillic acid, syringaldehyde	Godoy et al., 2002[82]
Eucalyptus maculata	Myrtaceae	elemol, β-eudesmol	Marsaro Junior et al., 2004[18]; Marinho et al., 2005[19]; Marinho et al., 2008[20]; Marinho et al., 2006[83]
Ricinus communis	Euphorbiaceae	Palmitic acid; ricin	Bigi et al., 2004[15]; Caffarini et al., 2008[84]; Cazal et al., 2009[85]
Dimorphandra mollis	Fabaceae	Astilbin	Cintra et al., 2005[86]
Raulinoa echinata	Rutaceae	Limonoid, limonexic acid	Biavatti et al., 2005[57]
Cedrela fissilis	Meliaceae	3*β*-acetoxicarapine limonoid, oleanolic acid, oleanic acid, cipadesin A, ruageanin A, cipadesin, khayasin T, febrifugin, mexicanolide	Bueno et al., 2005[60]; Leite et al., 2005[61]
Carapa guianensis	Meliaceae	6α-acetoxygedunin	Ambrozin et al., 2006[87]
Azadirachta indica	Meliaceae	Azadirachtin	Santos-Oliveira et al., 2006[62]; Brugger et al., 2008[63]
Helietta puberula	Rutaceae	Kokusagine, anthranilic acid, dictamnine	Almeida et al., 2007[20]

Plant species	Family	Identified substance	Reference
Ageratum conyzoides	Asteraceae	-	Ribeiro et al., 2008[88]
Mentha piperita	Lamiaceae	-	Ribeiro et al., 2008[88]
Simarouba versicolor	Simaroubaceae	4,5-dimethoxy-canthin-6-one, 5-methoxy-canthin-6-one	Peñaflor et al., 2009[22]
Citrus sp.	Rutaceae	Xanthyletin	Cazal et al., 2009[85]
Spiranthera odoratissima	Rutaceae	Furoquinolines, 2-arylquinolin-4-one, limonexic acid, limonin	Terezan et al., 2010[89]
Tithonia diversifolia	Asteraceae	-	Valderrama-Eslava et al., 2009[23]
Tabebuia vellosoi	Bignoniaceae	-	Souza et al., 2010[90]
Magonia pubescens	Sapindaceae	-	Souza et al., 2010[90]
Annona reticulata	Annonaceae	-	Souza et al., 2010[90]
Amburana acreana	Leguminosae	-	Souza et al., 2010[90]
Virola sebifera and Virola sp.	Myristicaceae	Sesamin and Epigalgavrin; (+)-sesamin, (-)-hinoquinin, (-)-kusunokinin	Bicalho et al., 2012[54]; Pagnocca et al., 1996[91]

Table 1. Plant species with toxic effects against leaf-cutting ants and/or their symbiotic fungi and the associated isolated substances.

3.1. *Sesamum indicum*

Crude extracts of the leaves, fruits, and seeds of sesame, *Sesamum indicum* L. (Pedaliaceae), were tested *in vitro* against the symbiotic fungus (*L. gongylophorus*) of *A. sexdens*, isolated from previously established nests. Bioassays were performed according to methodology developed by Pagnocca et al. (1990; 1996) [49,50]. The extracts were added to the culture medium described by Pagnocca et al. (1990) until reaching final concentrations between 7.5 and 60 mg/mL. Ten test tubes were used for each sample, with three replicates for leaf extracts and two replicates for the other extracts. Fungal growth was estimated macroscopically based on the surface area and density of the mycelium after 30-35 days of incubation. The control sample received the same amount of solvent, and the relative growth observed was characterised as follows: 5 + = growth equal to the control; 4 + = growth equivalent to 80% of the control; 3 + = growth equivalent to 60% of the control; 2 + = growth equivalent to 40% of the control; and 1 + = growth equivalent to 20% or less of the control. The crude extracts of sesame leaves, fruits, and seeds inhibited the growth of the symbiotic fungus, which suggests that this species produces compounds with antifungal proprieties (Tables 2 and 3).

Solvent	Final concentration (mg leaf dry weigh/mL)	Relative fungal growth (30-35 days)
Chloroform	7.5	5+
	15.0	5+
	30.0	4+
	60.0	2+
Methanol	7.5	5+
	15.0	5+
	30.0	4+
	60.0	2+
Chloroform+Methanol	30.0+30.0	2+
	60.0+60.0	<1+
Water	60.0	>5+

5+=growth identical to control; 4+=growth equivalent to 80% of control; 3+=growth equivalent to 60% of control; 2+=growth equivalent to 40% to control; 1+=growth equivalent to 20% of control of less. Source: Pagnocca et al., 1990 [49].

Table 2. Antifungal activity of sesame leaf extracts

Material and developmental stage	Final concentration mg dry weight/mL	Relative fungal growth (30-35 days)
L1 (leaves 30 days old)	60.0	2+
L2 (leaves 60 days old)	60.0	2+
L3a (green leaves 90 days old)	60.0	3+
L3b (yellow leaves 90 days old)	60.0	2+
Gfr (green fruit)	30.0	3+
RFr (ripe fruit)	30.0	2+
GS (green seed)	30.0	2+
RS (ripe seed)	30.0	2+

Control=5+

Table 3. Antifungal activity of chloroform extracts of sesame leaves, fruits, and seeds at different stages of development. Source: Pagnocca et al., 1990 [49].

In another study, Pagnocca et al. (1996) determined the number of bacteria and yeast in the organic matter within ant colonies reared in the laboratory with *Eucalyptus alba* Reinw. ex Blume (control) or *S. indicum* (experiment). Transparent plastic pots (2.5 L), connected to each other

through transparent tubes (1.5 cm of diameter), were used in these bioassays. In this setup, one chamber was used to supply leaves, a second chamber housed the fungal garden (sponge), and the third chamber contained the residues (waste) from the ants. Fresh leaves (10 to 20 grams) were offered at 48-h intervals after removal of the waste from the previous treatment. In the older sponges in nests treated with *Eucalyptus*, 1.4×10^5 bacterial colony forming units/g (CFU/g) were recorded, while the average in the waste deposits reached 7.3×10^7 CFU/g. The most probable numbers (MPNs) of yeast per gram of the material analysed were 1.3×10^5 and 2.2×10^4 MPN/g for older sponges and waste deposits, respectively, while in ant colonies treated with *S. indicum* leaves, these values were 3.3×10^7 CFU/g and 6.7×10^5 MPN/g. This increase in the numbers of bacteria and yeast led to visible changes in the colouration and humidity of the fungal sponges of nests treated with sesame, which resulted in fungal death.

The application of fractions of the extracts from sesame leaves at a 2.5 mg/mL concentration completely inhibited the development of the symbiotic fungus of the leaf-cutting ants, and 50% inhibition of fungal development was observed for some fractions at a 1.25 mg/mL concentration [51] (Table 4). Chromatographic analysis of the hexanic extracts of leaves revealed the presence of a mixture of tetradecanoic, hexadecanoic, octadecanoic, icosanoic, docosanoic, and 9,12,15-octadecatrienoic acids. Separation of the compounds in the mixture by fractionation resulted in a loss of or decrease in inhibitory activity against the fungus, indicating that the observed inhibition may be a consequence of the joint action of several compounds in the leaves, rather than of a single substance.

Fractions	Extracts		
	Hexane	**Dichloromethane**	**Methanol**
Hexane	-	[1.25]/(90)	[1.25]/(60)
	-	[2.50]/(NG)	[2.50]/(NG)
	[5.00]/50	[5.00]/(NG)	[5.00]/(NG)
Dichloromethane	-	[1.25]/(50)	-
	-	[2.50]/(NG)	-
	[5.00]/(90)	[5.00]/(NG)	[5.00]/(10)
Ethyl Acetate	[1.25]/(50)	[1.25]/(50)	[1.25]/(70)[2]
	[2.50]/(NG)	[2.50]/(NG)	[2.50]/(NG)
	[5.00]/(NG)	[5.00]/(NG)	[5.00]/(NG)
Methanol	[1.25]/(90)	[1.25]/(70)	[1.25]/(50)[2]
	[2.50]/(NG)	[2.50]/(NG)	[2.50]/(NG)
	[5.00]/(NG)	[5.00]/(NG)	[5.00]/(NG)
Acetic Acid	[1.25]/(100)	[1.25]/(100)	[1.25]/(100[2])
	[2.50]/(40)	[2.50]/(NG)	[2.50]/(NG)
	[5.00]/(10)	[5.00]/(NG)	[5.00]/(NG)

[1]Thirty days of incubation/25°C; 2dry mix; NG=no growth

Table 4. Fungal growth (%) of *Leucoagaricus gongylophorus* in culture medium containing different concentrations [mg/mL] of the hexane, dichloromethane, and methanol extracts from sesame. Control= (100). Source: Ribeiro et al., 1998 [51].

Extracts from ripe sesame seeds were tested to investigate their toxicity through contact with *A. sexdens* workers. Ripe seeds of *Sesamum indicum* L. (Pedaliaceae) were triturated and pressed, yielding sesame butter. A known mass of this sesame butter was macerated for three days three times at room temperature and then extracted with solvents of increasing polarity (dichloromethane and methanol), resulting in a dichloromethane crude extract (SD) and a methanol crude extract (SM). The SD crude extract was subjected to liquid chromatography in a vacuum syntherised plate funnel with silica gel as the stationary phase and eluents of increasing polarity, which yielded the following fractions: hexane (SD-H), dichloromethane (SD-D), ethyl acetate (SD-E), and methanol (SD-M). The SD-E fraction was produced through successive chromatographic columns, with silica as the stationary phase and hexane/dichloromethane/methanol as the eluent, in gradient mode. A total of 11 sub-fractions were obtained from this process, only four of which (A, B, C, D) contained a sufficient amount of material to be tested. At the tested concentrations, the same proportion as in the original SD-E fraction was maintained in the sub-fractions, and samples at double these concentrations were also tested (Figure 3). The SD-E sub-fractions were combined in amounts necessary to equal that of the original fraction. The seven sub-fractions (E-K) that were isolated in only small amounts were not tested. Tests were also performed in which the concentration of each sub-fraction was reduced by 50% in two combinations: A+B+C+D and A+B+C. To identify the compounds present in SD-E, hydrogen nuclear magnetic resonance (H NMR) and gas chromatography-mass spectrometry (GC-MS) were used. The results demonstrated that *A. sexdens* workers that received the crude dichloromethane extract from sesame seeds (SD) on their *pronoto* exhibited high mortality. This crude extract was then fractionated, and the ethyl acetate fraction (SD-E) was found to be responsible for the toxic effect. However, no toxicity was observed when the SD-E sub-fractions (A, B, C, and D) were tested in the same proportions as found in the original fraction (Table 5). These results could be explained by three hypotheses: 1) each isolated sub-fraction is only toxic at concentrations above the concentration found in the ethyl acetate fraction; 2) the sub-fractions are only toxic when combined through a synergistic effect between their components; and 3) toxic compounds are be present in the untested sub-fractions (E-K), which corresponded to 26.77% of the ethyl acetate fraction. Experiments were conducted to determine why the formicidal activity was lost. First, the authors doubled the concentration of each sub-fraction, and only one fraction, composed of triglycerides, was found to be toxic (Table 5). Then, when sub-fractions A, B, C, and D were combined, the formicidal effect reappeared, even at concentrations reduced to 50% of the original concentration (Table 6). A mixture containing 73.23% (A + B + C + D) of the ethyl acetate fraction contains chemical compounds that reduce the survival of *A. sexdens*. [52]

The results shown in Table 6 indicate that five of the 11 possible combinations of the SD-E sub-fractions were toxic to leaf-cutting ants (A + B + C + D; A + B + C; A + C + D; A + C; B + C), and all of the toxic combinations contained sub-fraction C, which was composed of diglycerides and furfuranic lignans (sesamin and sesamolin). The observed effects are likely due to the presence of lignin furfuranic, which is used as a synergistic factor in insecticides. However, sesamolin exhibited a biological activity that was five times stronger than that of sesamin. Moreover, sub-fraction D, which was composed only of sesamin, either had an in-

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Material	Concentration (mg mL^{-1})	% Mortality/Day										S_{50}
		1	2	3	6	8	10	14	17	21	25	
Control D	-	0	0	2	10	35	53	72	90	93	100	10a
SD	200	2	7	18	47	73	90	93	95	97	100	7c
SM	200	3	10	17	45	67	75	87	90	97	100	7c
Control H	-	0	5	7	23	32	57	75	78	85	100	9a
SD H	200	8	53	63	77	85	88	88	92	97	100	2c
Control D	-	5	5	15	37	62	78	85	97	97	100	7b
SD-D	200	0	5	5	27	53	63	78	82	92	100	8a
Control E	-	2	3	5	27	58	68	88	95	97	100	8a
SD-E	200	0	5	7	23	32	57	75	78	85	100	9a
Control M	-	27	47	57	67	70	75	88	93	100	-	3c
SD-M	200	0	5	5	27	53	63	78	82	92	100	8a
Control E	-	0	5	5	20	37	72	88	92	98	100	9a
A	57	2	10	10	37	62	65	77	82	92	100	8a
A	14	0	5	7	23	32	57	75	78	85	100	9a
Control E	-	23	38	53	83	87	87	97	98	98	100	3c
B	53	0	5	8	18	35	53	70	80	85	100	10a
B	106	0	5	5	27	53	63	78	82	92	100	10a
C	25	2	2	2	28	65	83	95	97	100	-	8b
C	50	0	2	8	25	42	60	83	88	90	100	10a
Control E	-	0	5	7	23	32	57	75	78	85	100	9a
D*	12	0	2	3	18	30	63	85	93	98	100	9a
D*	24											

S_{50}= Survival median 50%. Different letters after the S_{50} values indicate a significant difference according to the log-rank test (b=0.01>p<0.05; c=p<0.01). Concentrations: A= 57 mg mL^{-1}; B=53 mg mL^{-1}; C=25 mg mL^{-1}; D*= 12 mg mL^{-1}; ( )*= concentration reduced to 50%

Table 5. Toxicity of SD-E sub-fraction combinations in *Atta sexdens* workers. Source: Morini et al., 2005 [52].

hibitory effect on the action of other sub-fractions (B + C + D; C + D) or was unable to modify their actions (A + D; B + D), showing that the factor responsible for the synergistic toxic effect of sesame seeds is either sesamolin or the combination of sesamin + sesamolin, rather than sesamin alone [52] (Table 6).

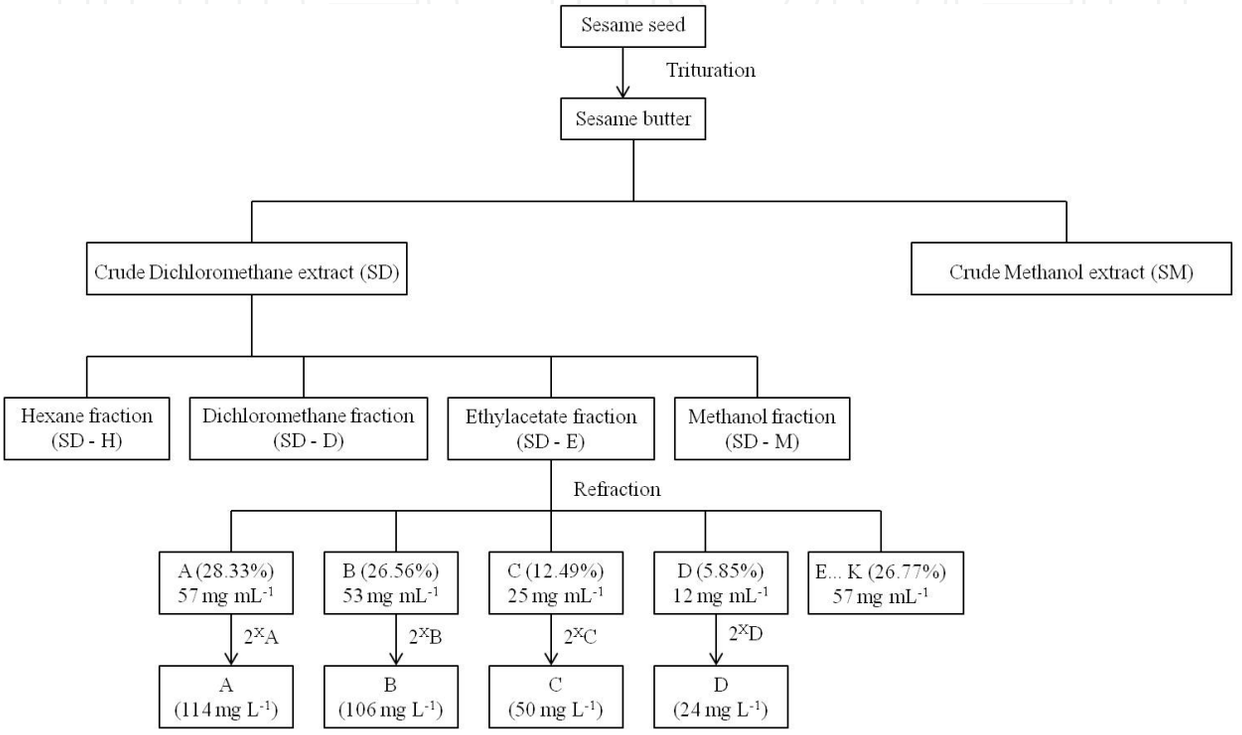

Figure 3. Diagram showing the procedure for obtaining crude extracts, fractions, and sub-fractions from sesame seeds (*S.indicum*) and the sequence of topical application on *A. sexdens rubropilosa* workers. Source: Morini et al., 2005 [52].

The efficiency of different commercial chlorpyrifos- sulfluramid- and fipronil-based formicidal baits as well as others that are manually manufactured using the leaves (15%) and seeds (10%, 20% and 30%) of *S. indicum* against *A. sexdens* Forel. control were assessed in the field. The nest activity was monitored at 30, 60, 90, and 150 days after treatment. The most efficient baits were sulfluramid- and fipronil-based, followed by the formulation derived from sesame leaves (15%). The sulfluramid- and fipronil-based baits caused colony activity to cease at 30 days, while the sesame leaf-based baits (15%) resulted in an 80% inhibition of activity at 90 days, confirming that *S. indicum* has great potential for the development of new products to control leaf-cutting ants [53].

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Sub–fraction combination	% Mortality/Day										S_{50}
	1	2	3	6	8	10	14	17	21	25	
Control	0	0	2	10	35	53	72	90	93	100	10a
A+B+C+D*	2	7	18	47	73	90	93	95	97	100	7c
(A+B+C+D*)*	3	10	17	45	67	75	87	90	97	100	7c
Control	0	5	7	23	32	57	75	78	85	100	9a
A+B+C	8	53	63	77	85	88	88	92	97	100	2c
(A+B+C)*	5	5	15	37	62	78	85	97	97	100	7b
Control	0	5	5	27	53	63	78	82	92	100	8a
A+B+D*	2	3	5	27	58	68	88	95	97	100	8a
Control	0	5	7	23	32	57	75	78	85	100	9a
A+C+D*	27	47	57	67	70	75	88	93	100	-	3c
Control	0	5	5	27	53	63	78	82	92	100	8a
B+C+D*	0	5	5	20	37	72	88	92	98	100	9a
A+B	2	10	10	37	62	65	77	82	92	100	8a
Control	0	5	7	23	32	57	75	78	85	100	9a
A+C	23	38	53	83	87	87	97	98	98	100	3c
A+D*	0	5	8	18	35	53	70	80	85	100	10a
Control	0	5	5	27	53	63	78	82	92	100	10a
B+C	2	2	2	28	65	83	95	97	100	-	8b
B+D*	0	2	8	25	42	60	83	88	90	100	10a
Control	0	5	7	23	32	57	75	78	85	100	9a
C+D*	0	2	3	18	30	63	85	93	98	100	9a

S_{50}= Survival median 50%. Different letters after the S_{50} values indicate a significant difference according to the log-rank test (b=0.01>p<0.05; c=p<0.01). Concentrations: A= 57 mg mL^{-1}; B=53 mg mL^{-1}; C=25 mg mL^{-1}; D*= 12 mg mL^{-1}; ( )*= concentration reduced to 50%

B=53 mg mL^{-1}; C=25 mg mL^{-1}; D*= 12 mg mL^{-1}; ( )*= concentration reduced to 50%

Table 6. Toxicity of SD-E sub-fraction combinations in *Atta sexdens* workers. Source: Morini et al., 2005 [52].

3.2. *Virola sebifera*

Phytochemical analysis of the leaves of *Virola sebifera* Aubl. (Myristicaceae) resulted in the isolation of three lignans, (+)-sesamin (1), (-)-hinoquinin (2), and (-) – kusunokinin (3) (Figure 4), and three flavonoids, quercetin-3-O-α-L-rhamnoside, quercetin-3-O-β-D-glucoside, and quercetin-3-methoxy-7-O-β-D-glucoside. Techniques such as high-speed counter-current chromatography and high-performance liquid chromatography were employed in this process. The isolated substances were added to the artificial diet and tested against *A. sexdens* leaf-cutting ants at a concentration of 200 or 400 μg mL^{-1}. Diets (0.4-0.5 g per dish) treated with the compounds (experimental treatment) or without (control) were offered daily in a small plastic cap. The percentage of survival was plotted as a function of time in a survival curve that was then used to calculate the median survival time (S_{50}, the time at which 50% of the ants in each experiment remained alive). The lignin (-) - kusunokinin (3) resulted in 90% mortality of *A. sexdens* workers after 25 days of monitoring compared to the controls fed with an untreated diet. Although the other substances did not show biological activity against the ants, the (+)-sesamin (1), (-)-hinoquinin (2) and (-)-kusunokinin (3) lignans inhibited the growth of the symbiotic fungus by 74%, 72%, and 100%, respectively [54] (Figure 4).

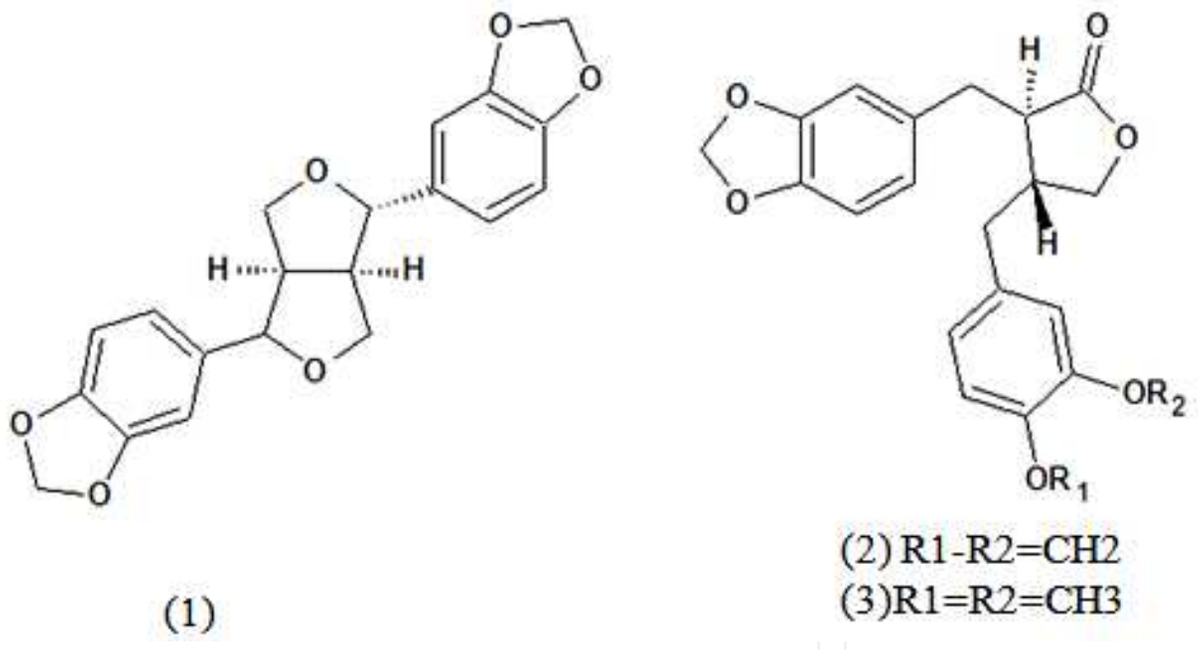

Figure 4. Chemical structures of the compounds isolated from *Virola sebifera,* (+)-sesamin (1), (-)-hinoquinin (2), and (-) – kusunokinin (3). Source: Bicalho et al., 2012 [54].

3.3. *Canavalia ensiformis*

In vitro tests showed inhibitory effect on the symbiotic fungus of a hexanic extract of *Canavalia ensiformis* (L.) DC. (Fabaceae) leaves, applied at a 1,000 μg mL^{-1} concentration. This extract was fractionated by column chromatography using silica gel as the stationary phase. A total of 11 fractions were obtained and used in fungal bioassays at a concentration of 500 μg mL^{-1}. Only

one fraction (fraction 9) was active; all fractions were esterified with diazomethane and analysed by gas chromatography-mass spectrometry (GC-MS) to identify the active components. The main compounds identified in the active fraction were long-chain saturated fatty acids. In these experiments, it was not possible to identify which of the fatty acids was responsible for the fungicidal action. However, comparison of the different fractions showed that the fatty acids with chains containing 11, 17, 19, 22, and 23 carbon atoms were likely the most active (Table 7), as the fractions in which these fatty acids were not among the major components showed no fungicidal activity [55].

Fraction	Major compounds	Minor compounds
9*	C_{11}, C_{16}, C_{17}, C_{18}, C_{19}, C_{20}, C_{22}, C_{23}, C_{24}, C_{26}	C_8, C_{14}, C_{15}, C_{21}, C_{25}, C_{27}, C_{28}, C_{30}
2	C_{10}, C_{16}, C_{18}, C_{24}, C_{26}	C_{14}, C_{15}, C_{17}, C_{22}, C_{23}, C_{25}, C_{28}, C_{29}
3	C_{16}, C_{18}	C_{10}, C_{14}, C_{15}
4	C_{16}, C_{20}	C_{18}, C_{21}, C_{22}
5	C_{16}	C_{12}

*Active fraction

Table 7. Fatty acids found in *Canavalia ensiformis*. Source: Monteiro et al., 1998 [55].

3.4. *Raulinoa echinata*

Phytochemical analyses of the roots of *Raulinoa echinata* R.S.Cowan (Rutaceae) resulted in the isolation and identification of the following limonoids: fraxinellone, fraxinellonone, and epoxy-fraxinellone. Limonexic acid was isolated from the stem of the plant. The toxicity of the compounds against *A. sexdens* was determined in ingestion bioassays according to the protocol described by Bueno et al. (1997) [56]. The ants in the treatment groups received a diet enriched with epoxy-fraxinellone or limonexic acid at a concentration of 200 μg mL^{-1}. Control ants were fed with a component-free diet. Over 25 days, the number of dead ants in each Petri dish was counted, the survival curve of the leaf-cutting ants in each treatment was estimated, and their average longevity was calculated. Limonexic acid (4) (Figure 5) reduced the longevity of *A. sexdens* considerably (11 days) compared to the control (22 days) [57]. *R. echinata* was also able to produce substances that were active against the symbiotic fungus of the leaf-cutting ants; several furoquinoline alkaloids (skimmianine (5), kokusaginine (6), maculine (7) and flindersiamine (8)) and quinolones (2-n-Nonyl-4-quinolone (9), 1-Methyl-2-n-nonyl-4-quinolone (10), 1-Methyl-2-phenyl-4-quinolone (11)) (Figure 6; Table 8) that exhibited fungicidal activity against *L. gongylophorus* were isolated from extracts of its stems and leaves [58].

3.5. *Helietta puberula*

Methanolic, hexanic, and dichloromethane extracts obtained from the stems, leaves, and branches of *Helietta puberula* R. E. Fr. (Rutaceae) were tested against *A. sexdens* workers and the symbiotic fungus of this ant species. Experimental diets were prepared by mixing plant

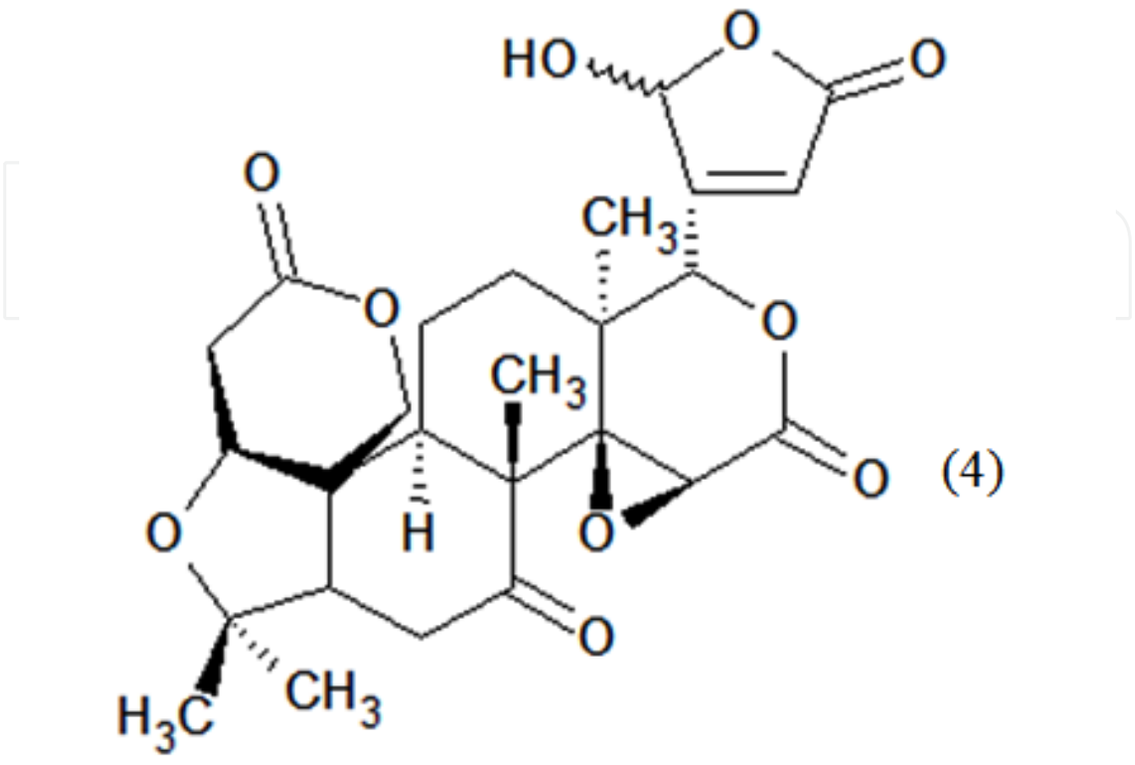

Figure 5. Limonexic acid isolated from *Raulinoa echinata* stems. Source: Biavatti et al., 2005 [57]

(5) R^1=H, R^2=R^3=OMe
(6) R^1=R^2=OMe, R^3=H
(7) R^1=R^2=OCH_2O, R^3=H
(8) R^1=R^2=OCH_2O, R^3=OMe

(9) R^1=H, R^2=*n*-nonyl
(10) R^1=Me, R^2=*n*-nonyl
(11) R^1=Me, R^2=phenyl

Figure 6. Compounds identified as skimmianine (5), kokusagine (6), masculine (7), flindersiamine (8), 2-n-Nonyl-4-quinolone (9), 1-Methyl-2-n-nonyl-4-quinolone (10), and 1-Methyl-2-phenyl-4-quinolone (11) based on comparison with spectral data presented in the literature. Source: Biavatti et al., 2002 [58]

Extract/fraction/compound	% growth inhibition of *L. gongylophorus*				
	μg mL^{-1} 50	100	250	500	1000
MSE[a]	-	-	-	-	80
Skimmianine (1)	60	80	NT	NT	NT
Kokusagine (2)	20	100	NT	NT	NT
Maculine (3)	10	50	NT	NT	NT
Flindersiamine (4)	-	50	NT	NT	NT
MLE[c]	-	-	-	-	80
MLE (hexane fraction)				80	100
2-n-Nonyl-4-quinolone (5)	20	50	NT	NT	NT
1-Methyl-2-n-nonyl-4-quinolone (6)	-	-	-	-	-
1-methyl-2-phenyl-4-quinolone (7)	NT	NT	NT	NT	NT

[a]MSE: Methanol stem extract, [b]NT: not tested, [c]MLE: methanol leaf extract

Table 8. Evaluation of the growth inhibitory activity of crude extracts, fractions, and compounds of *Raulinoa echinata*

material (crude extract, partially purified extract, or pure compound) and the basic formula described by Bueno et al. (1997) [56]. The final concentrations of crude extracts, fractions, and isolated substances from *H. puberula* in the diet were 2.0, 1.6, and 0.3 mg mL^{-1}, respectively. Blocks of 0.4 g of the experimental diets per plate (control or experimental) were offered daily to the workers. Evaluations were conducted over 25 days, and the number of dead ants was recorded daily. The following substances were isolated from *H. puberula:* anthranilic acid (12), flindersiamine (13), dictamnine (14), kokusaginin (15), maculine (16), and sitosterol. The anthranilic acid, kokusaginine, and dictamnine resulted in 90%, 86%, and 88% mortality, respectively, compared with 68% mortality in the control. The substances anthranilic acid, kokusaginine, masculine, and dictamnine caused fungal inhibition (≥80%) at a concentration of 0.1 mg mL^{-1} [21] (Figure 7).

3.6. *Eucalyptus* sp.

Leaf-cutting ants may exhibit behavioural changes when exposed to plant extracts; Anjos and Santana (1994) [59] observed bites and mutilations among *A. sexdens* and *A. laevigata* nestmates subjected to contact with leaves of four *Eucalyptus* sp. belonging to the family Myrtaceae. With the aim of isolating and identifying the compounds responsible for these changes, *E. maculate* leaves were subjected to extraction with hexane, followed by chromatographic fractionation, resulting in the isolation of six active sesquiterpenes (elemol, β-eudesmol, α-eudesmol, guaiol, hinesol, and γ-eudesmol).

Fragments of filter paper in a rectangle, square, or triangle shape were prepared and (a) impregnated with solvent alone as a control (square), (b) left blank (rectangle), or (c) impregnated with the treatment to be tested using 100 μL of the extract solution or pure compound (triangle).

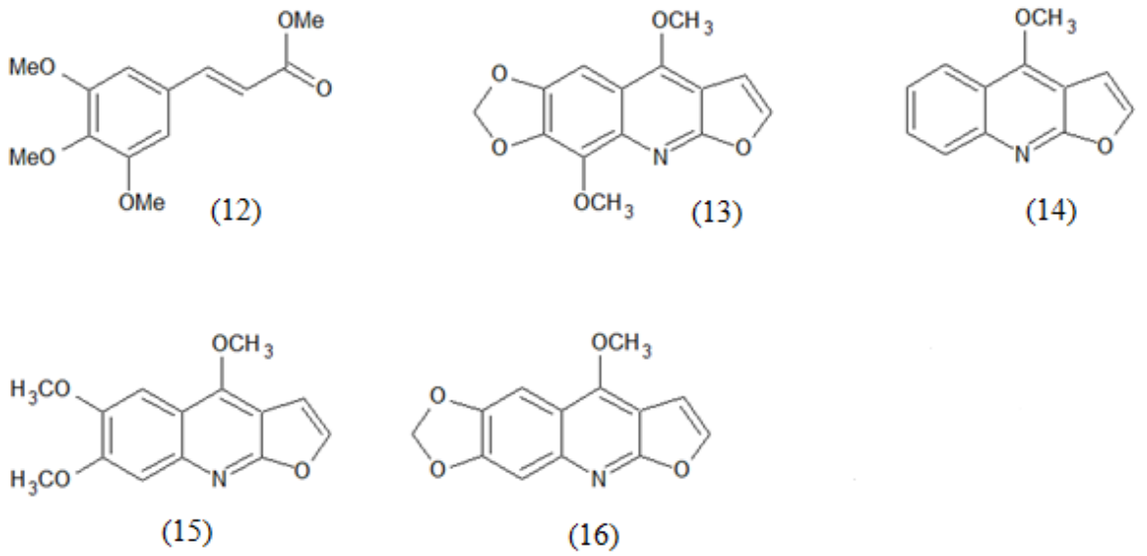

Figure 7. Molecular structures of substances from *Helietta puberula*: anthranilic acid (12), flindersiamine (13), dictamnine (14), kokusaginine (15), and maculine (16). Source: Almeida et al., 2007 [21].

After solvent evaporation, two of the filter paper fragments of each of the three different geometric shapes were placed on three glass slides, which were then transferred to the colonies. Monitoring was performed for 30 minutes after placement of the filter paper, and the number of groups of attackers, the number of ants in each group, and the number of mutilated ants in each group were counted. Elemol (17) and β-eudesmol (18) (Figure 8) were the most active ingredients, and the latter substance was associated with greater numbers of groups of attackers (84.2) and mutilated ants (285.8). After contact with the filter paper impregnated with β-eudesmol, the ants exhibited alarm behaviour and held their mandibles open. When encountering nestmates that had previously contacted the filter paper, they touched their antennas and then attacked each other, frequently on the legs, but also on other parts of the body [18].

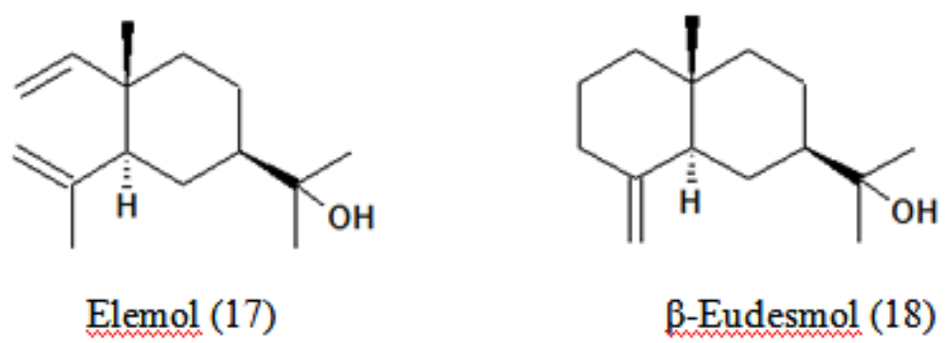

Figure 8. Chemical structures of the sesquiterpenes identified in the most active fraction of the *Eucalyptus maculate* leaf extract. Source: Marsaro Junior et al., 2004 [18].

Upon analysis, the composition of the chemical profile of the cuticles of the workers that had contact with β-eudesmol was different than that found in the other workers. (E)-β-farnesene, busenol, and (E,E)-farnesol were present in the cuticles of ants exposed to β-eudesmol [20]. The changes in the composition of the cuticle interfered in the process of recognition between nestmates. The ants triggered an alarm behaviour when they did not recognise the workers exposed to β-eudesmol.

3.7. *Cedrela fissilis*

The survival of *A. sexdens* workers was significantly reduced when they were fed diets containing hexane or dichloromethane-soluble extracts of the root and leaves of *Cedrela fissilis* Vell. (Meliaceae). These extracts and those derived from fruits and branches, which were hexane- or dichloromethane-soluble, respectively, also inhibited the growth of the *L. gongylophorus* fungus [60,61].

The limonoid 3β-acetoxicarapin and the triterpenes oleanoic and oleanonic acid were isolated from roots of *C. fissilis*. These compounds and six other mexicanolide-type limonoids (cipadesin A, ruageanin A, cipadesin, khayasin T, febrifugin, and mexicanolide) that were previously isolated from *Cipadessa fruticosa* Blume exhibited insecticidal activity against *A. sexdens* leaf-cutting ants. The median survival period (S_{50}) was significantly different from that of the control, confirming activity against *A. sexdens* [61] (Table 9).

Compounds	Days										Survival median (S_{50})/days
	1	2	3	6	8	10	14	17	21	25	
1- cipadesin B	0	0	6	22	40	58	76	88	92	98	9[a]
2- swietemahonolide	0	0	4	40	54	58	82	90	96	98	8[a]
3- 3β-acetoxycarapin	0	2	12	34	50	76	98	100	-	-	8[a]
4- oleanolic acid	0	0	10	52	70	90	100	-	-	-	6[a]
5c- oleanonic acid	0	0	8	38	60	86	100	-	-	-	8[a]
6- cipadesin A	0	2	8	34	46	72	100	-	-	-	9[a]
7- ruageanin A	0	4	18	50	62	74	96	98	98	100	6[a]
8- cipadesin	0	8	12	46	68	76	100	-	-	-	7[a]
9- khayasin T	0	4	10	54	72	86	98	100	-	-	6[a]
10- febrifugin	0	2	6	38	58	70	88	94	98	100	7[a]
11- mexicanolide	0	4	16	50	60	70	100	-	-	-	6[a]
Control (ethyl acetate)	0	0	6	22	36	50	74	90	94	96	10

[a]Significant difference according to the log-rank test ($p<0.05$).

Table 9. Mortality (%) of *Atta sexdens* workers fed on compounds 1-11 at a concentration of 100 μg mL^{-1}. Source: Leite et al., 2005 [61].

3.8. *Azadirachta indica*

Seeds of *Azadirachta indica* were tritured and pressed, yielding a neem paste. After one week, the floating material was isolated, which was referred to as crude extract of neem oil. A known mass of the remaining material, referred to as crude extract of seed neem paste, was macerated for three days three times at room temperature and extracted with solvents of increasing polarity (hexane, dichloromethane and methanol), resulting in three crude extracts. When incorporated in an artificial diet, the crude extract of neem seed oil caused significant toxicity to *A. sexdens* workers at all of the concentrations tested. The survival of the ants was significantly reduced in the diets containing the neem seed paste hexane extract at concentrations of 10 and 20 μg mL^{-1}, the dichloromethane extract at all concentrations tested (2, 10, and 20 μg mL^{-1}), and the methanol extract at concentrations of 10 and 20 μg mL^{-1}.

There was a negative relationship between the neem oil concentration and the frequency of contact of ants with the artificial diet. The lowest frequency of contact was obtained with the highest concentration tested (30 μg mL^{-1}). Moreover, the initial contact with the diet was dependent on the presence of neem. Thus, the period required for the ants to feed on the artificial diet for the first time was 8 seconds in the control, 4 minutes and 36 seconds at a concentration of 5 μg mL^{-1}, 19 minutes at a concentration of 10 μg mL^{-1}, and 55 minutes at a concentration of 30 μg mL^{-1}. Some changes in the behaviours of the ants were observed when the workers contacted the diets containing neem seed oil. Contact between the antenna or legs and the diet caused instantaneous retraction of these body parts. The ants positioned themselves offensively with open mandibles and performed self-grooming. The workers that cleaned themselves by licking showed symptoms of intoxication, such as slow movements, disorientation, and prostration [62].

The hexanic extract of *A. indica* neem was tested against *Acromyrmex rugosus* F. Smith (Formicidae) workers. Two colonies of *A. rugosus* were used, and from each colony, 30 groups of 20 workers each were isolated. A citrus pulp containing neem at concentrations of 0.1, 1.0, and 10% was offered to these groups. In the treatments, pastes composed of hexanic extracts of neem (from leaves, branches and seeds) were prepared with the following composition: pure glucose (10%), citrus pulp powder and soybean oil (10%). The sulfluramid treatment was offered in the form of a paste, in which 0.3% sulfluramid was dissolved in 10% soybean oil and mixed with citrus pulp powder and 10% pure glucose. The positive control was prepared in the same manner, but the active sulfluramid was not added to the paste. Treatments were performed with 5 g of paste per replicate, which was removed from the jars after 48 hours. Monitoring lasted five minutes and was performed immediately, 30 minutes, and 24 hours after treatment. The relative frequencies of each of the workers' behaviours and the number of deaths were recorded. High mortality was observed within the first 24 h in the treatments with neem (>20 workers) compared to the control (5 workers), which is not the slow type of action desired for formicides. The delayed action of the active ingredients in formicide formulations is an essential feature because colonies of leaf-cutting ants are very populous, and the control of their nests depends on contamination of all individuals. If ants detect that the presented substrate is not adequate, they will stop carrying it and can even remove parts of the symbiotic fungus contaminated with this substrate and isolate it in waste chambers [63].

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3.9. *Simarouba versicolor*

The dichloromethane-soluble fraction of methanolic extracts of the leaves, stems, and branches of *Simarouba versicolor* St. Hill (Simaroubaceae) was tested *in vitro* on ants through ingestion bioassays and with the symbiotic fungus in culture medium. The median survival period for workers was significantly reduced (S_{50}=4 days) compared to the control (S_{50}=16 days), and 100% inhibition of *L. gongylophorus* growth was observed. From these fractions, two alkaloids were isolated, 4,5-dimethoxy-canthin-6-one (19) and 5-methoxy-canthin-6-one (20) (Figure 9), both of which were toxic to the symbiotic fungus and completely inhibited growth at a concentration of 0.1 mg mL^{-1}. However, only the alkaloid 5-methoxy-canthin-6-one reduced the median survival period of the workers from 14 days (control) to seven days at a 0.3 mg mL^{-1} concentration (Table 10). The triterpenes isolated from the other extracts of the plant (lupenone and lupeol) showed no deleterious effects on the leaf-cutting ants of the symbiotic fungus [22].

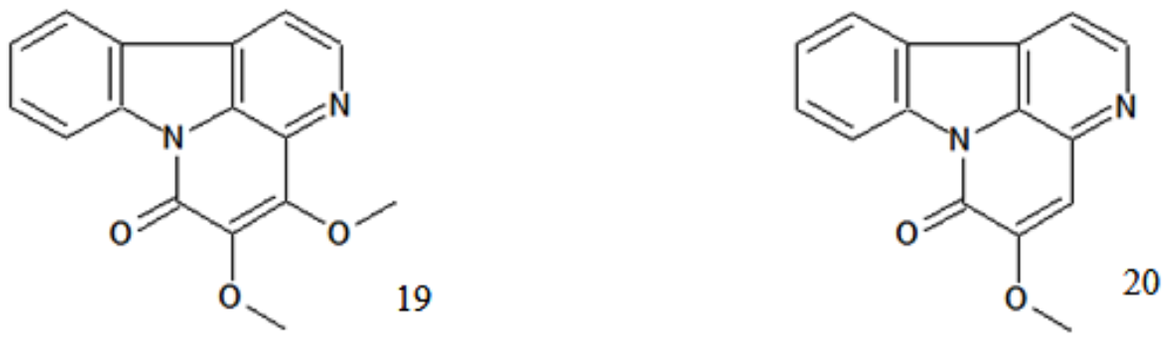


Figure 9. Chemical structures of substances isolated from *Simarouba vesicolor*: 4,5-dimethoxycanthin-6-one (19) and 5 methoxycanthin-6-one (20). Source: Peñaflor et al., 2009 [22].

Treatment[1]	Day of experiment										
	1	2	3	6	8	10	14	17	21	25	S_{50} [2]
Control	0	2	8	22	30	38	52	62	74	76	14a
Lupenone	4	4	6	12	24	30	46	54	64	76	16a
4,5-dimethoxycanthin-6-one	2	2	8	34	36	48	56	58	68	82	13a
Lupenol	2	2	10	26	26	28	34	36	44	54	19a
5-methoxycanthin-6-one	0	0	10	46	52	56	76	78	80	88	7b

[1]Isolated substances at a concentration of 0.3 mg mL^{-1};

[2]S_{50}=Survival median 50%. Different letters after the S_{50} values indicate a significant difference according to the log-rank test. Different letters after the S_{50} values compared to the respective control indicate a significant difference according to the log-rank test (P<0.05). Source: Peñaflor et al., 2009 [22].

Table 10. Toxicity (% mortality and S_{50}) of substances isolated from *Simarouba versicolor* in *Atta sexdens* workers.

3.10. *Ageratum conyzoides*

An assessment of the formicidal activity of a hexanic extract from the leaves of goatweed, *Ageratum conyzoides* L. (Asteraceae), against leaf-cutting ants was performed using the acetone-diluted extract at a concentration of 1.0 mg mL^{-1}. Each worker was topically treated with 1.0 μL of this solution, which was applied on the pronoto of the insect. In the control treatment, the insects were treated with an equal volume of pure acetone. The numbers of living and dead individuals were counted 24 and 48 hours after treatment. The crude extract of goatweed caused increased mortality of *Atta laevigata* F. Smith (Hymenoptera: Formicidae) and *Atta subterraneus subterraneus* Forel (Hymenoptera: Formicidae) workers. The goatweed extract was then fractionated, resulting in the isolation of the compound coumarin. Coumarin was tested against ants at different concentrations (0.5, 4.0, 7.0, 16.0, 50.0, and 100.0 mg mL^{-1} in acetone) to determine its toxicity among the two species of leaf-cutting ants. The median lethal concentration (LC_{50}) decreased (10.9-fold) with increased application time for *A. subterraneus subterraneus*. The LC_{50} was 55.42 mg mL^{-1} at 24 hours and decreased to 5.07 mg mL^{-1} at 48 hours. For *A. laevigata*, the LC_{50} decreased 1.8-fold, from 23.20 mg mL^{-1} at 24 hours to 12.70 mg mL^{-1} at 48 hours. Thus, coumarin is a potential agent for ant control in the form of granulated attractive baits because it has a delayed insecticidal effect [64].

3.11. *Ricinus communis*

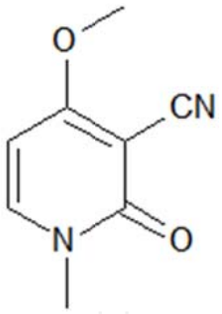
Dry *R. communis* leaves (2 kg) were ground in a Willey mill, and crude extracts were prepared via sequential maceration (3 litres for 7 days for each solvent) with hexane (24.8 g of extract), dichloromethane (32.8 g), ethyl acetate (18.8 g), methanol (54.0 g), and water. With the exception of the water extract, all extracts were subjected to chromatography on silica gel 60 as the stationary phase under vacuum (0.040-0.063 mm, 400 g; column with a sinterised filter in the bottom, internal diameter 10 cm, length 25 cm) with hexane, dichloromethane, ethyl acetate, and methanol (1 litre each) as eluents, yielding four fractions for each extract. The water extract was not fractionated. A portion of the methanol fraction of the hexane extract was refractionated, yielding 12 fractions (MFHE 1-12). These extracts were tested against the symbiotic fungus according to the methodology of Pagnocca et al. (1990) [49]. The sub-fractions MFHE-6, MFHE-9, and MFHE-10 inhibited fungal growth by 80% at a concentration of 0.5 mg mL^{-1}. The same result was observed for the MFHE-11 sub-fraction at a 1.0 mg mL^{-1} concentration. Sub-fraction MFHE-9 contained a mixture of two glycosidic steroids (β-sitosterol-3-*O*-*β*-D-glucoside and stigmasterol-3-*O*-*β*-D-glucoside) and fatty acids (decanoic, myristic, pentadecanoic, palmitic, heptadecanoic, estearic, eicosanoic, docosanoic, tricosanoic, and tetracosanoic acids). Among the above-mentioned compounds, only palmitic acid exhibited antifungal activity and inhibited the growth of the symbiotic fungus by 80% (Table 11).

The methanolic fraction of the dichloromethane-soluble extract of *R. communis* leaves was also re-fractionated, resulting in the isolation of ricin (21) (Figure 10) and monoglyceride (1-palmitic acid glycerol ester). Ricin caused significant death of *A. sexdens* workers when added to their artificial diets. The median survival periods (S_{50}) were 6.93 and 5.27 days at 0.2 and 0.4 mg mL^{-1}, respectively, compared to 10.82 days in the control. However, the effect on mortality was

Acid	%
Decanoic	1.2
Myristic	0.5
Pentadecanoic	6.4
Palmitic	81.0
Heptadecanoic	0.3
Estearic	6.6
Eicosanoic	1.1
Docosanoic	0.2
Tricosanoic	0.7
Tetracosanoic	0.2

Table 11. Activity of fatty acids present in the methanol fraction of hexane extracts from *Ricinus communis* leaves (MFHE) against *Leucoagaricus gongylophorus*. Source: Bigi et al., 2004 [15].

dose dependent. Symptoms of intoxication could be perceived after 24 hours and consisted of a reduction or cessation of movement, followed by disorientation, lack of coordination, and death [15].



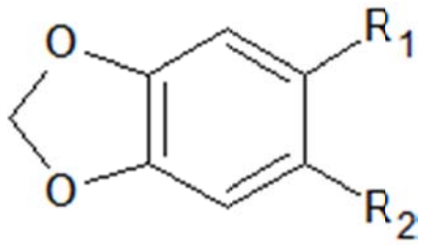
(21)

Figure 10. Ricinin isolated from leaf extracts of *Ricinus communis.* Bigi et al., 2004 [15].

3.12. Synthetic analogues of plant origin

The development of the symbiotic fungus *L. gongylophorus* is inhibited *in vitro* by synthetic compounds containing a piperonyl group: 1-(3,4-methylenedioxybenzyloxy)methane (22); 1-(3,4-methylenedioxybenzyloxy)ethane (23); 1-(3,4-methylenedioxybenzyloxy)butane (24); 1-(3,4-methylenedioxybenzyloxy)hexane (25); 1-(3,4-methylenedioxybenzyloxy)octane (26); 1-(3,4-methylenedioxybenzyloxy)decane (27); and 1-(3,4-methylenedioxybenzyloxy)dodecane (28) (Figure 11). Moreover, *A. sexdens* workers fed daily with an artificial diet containing these compounds showed high mortality compared to controls. The inhibition of fungal growth increased with the number of carbon atoms in the lateral chain, which varied from 1 to 8

(substances 22 to 26). Compounds containing 10 or 12 carbon atoms in the lateral chain did not inhibit fungal growth (substances 27 and 28) (Figure 11). Compound 26, 1-(3,4-methylenedioxybenzyloxy)octane, was the most active and inhibited fungal development by 80% at 15 μg mL^{-1}. In workers, a toxic effect was caused by compound 26 (C8); this effect increased with an increase in the number of carbon atoms in the lateral chains (C10 and C12). Thus, at the same concentration (100 μg mL^{-1}), the mortality rates after eight days of ingestion were 82%, 66%, and 42% under treatment with 1-(3,4-methylenedioxybenzyloxy)decane (compound 28), 1-(3,4-methylenedioxybenzyloxy)dodecane (compound 27), and compound 26, respectively, while for piperonyl butoxide, the observed mortality was 68%. The last compound, which is known as a synergistic insecticide, inhibited the symbiotic fungus with an intensity that was statistically similar to that observed for synthetic compound 26. The results indicate that a formulation can be designed to attack both ants and their symbiotic fungus; such a formulation could represent an advantage over the chemical products used for leaf-cutting ant control, which are directed only towards the ants [65].



22- $R_1=CH_2OCH_3$, $R_2=H$

23- $R_1=CH_2OCH_2CH_3$, $R_2=H$

24- $R_1=CH_2OCH_2(CH_2)_2CH_3$, $R_2=H$

25- $R_1=CH_2OCH_2(CH_2)_4CH_3$, $R_2=H$

26- $R_1=CH_2OCH_2(CH_2)_6CH_3$, $R_2=H$

27- $R_1=CH_2OCH_2(CH_2)_8CH_3$, $R_2=H$

28- $R_1=CH_2OCH_2(CH_2)_{10}CH_3$, $R_2=H$

29- $R_1=CH_2(OCH_2CH_2)_2O(CH_2)_3CH_3$, $R_2=CH_2CH_2CH_3$

Figure 11. Structures of the synthesised compounds 1-(3,4-methylenedioxybenzyloxy)methane (22); 1-(3,4-methylenedioxybenzyloxy)ethane (23); 1-(3,4-methylenedioxybenzyloxy)butane (24); 1-(3,4-methylenedioxybenzyloxy)hexane (25); 1-(3,4-methylenedioxybenzyloxy)octane (26); 1-(3,4-methylenedioxybenzyloxy)decane (27); 1-(3,4-methylenedioxybenzyloxy)dodecane (28); and the commercial compound piperonyl butoxide (29). Victor et al., 2001 [65].

Several studies have suggested that the amides found in species of the *Piper* genus show potential for insecticidal use due to their effectiveness and knockdown effects. Therefore, the natural amides N-pyrrolidine-3-(4,5-methylenedioxyphenyl)-2-(E)-propenamide and N-piperidine-3-(4,5-methylenedioxyphenyl)-2-(E)-propenamide, found in the roots of *Piper piresii* Yunck (Family: Piperaceae), were used as a model for the synthesis of analogous amides.

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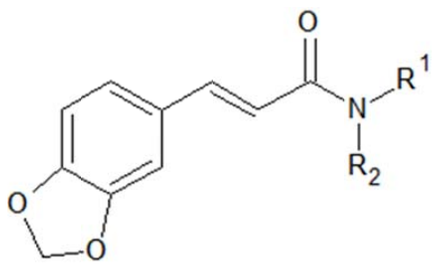
The 3-(3,4-methylenedioxyphenyl)-2-(E)-propenamide (30) portion was maintained, and only groups R_1 and R_2 linked to the nitrogen (Figure 12) were altered. Thus, nine amides were synthesised, and the yield varied between 36 and 86% (Table 12; Figure 13). Compounds 3 (S_{50}= 11 days) and 8 (S_{50}=7.5 days) significantly reduced the median survival period (S_{50}) for workers compared to the control (S_{50}= 14 days) at 100 μg mL^{-1} when added to the artificial diet offered daily. Compounds 1, 2, 4, 5, 6, 7, and 9 had no effect on the median survival period at any of the concentrations tested (25, 50, and 100 μg mL^{-1}). At 100 μg mL^{-1}, compounds 1, 2, and 3 completely inhibited fungal growth, and partial inhibition was observed for compounds 4 (80%), 5 (40%), and 6 (20%), while compounds 7, 8, and 9 had no effect on the growth of the symbiotic fungus [66].



(30)

Figure 12. E)-3-(3,4-methylenedioxyphenyl)-2-propenamide group. Source: Pagnocca et al., 2006 [66].

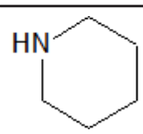
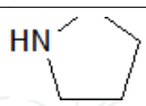
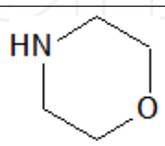
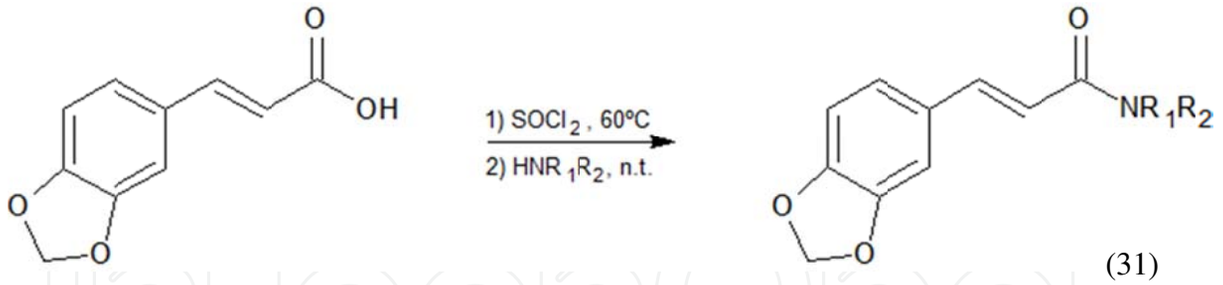
Amide	Amine	NR_1R_2	Yield (%)	Eluent (hexane/ethyl acetate)
1	Piperidine		44	4:6
2	Diethylamine	$N(CH_2CH_3)_2$	42	6:4
3	Pyrrolidine		68	1:1
4	2-Methylbutylamine	$NHCH_2CH(CH_3)CH_2CH_3$	38	7:3
5	Morpholine		40	5.5:4.5
6	Aniline	NHC_6H_5	39	5.5:4.5
7	Disopropylamine	$N[CH(CH_3)_2]_2$	36	2:1
8	Benzylamine	$NHCH_2C_6H_5$	36	4:1
9	Dicyclohexylamine	$N(C_6H_{11})_2$	86	7.5:2.5

Table 12. Amines, the respective yields of the amides, and the eluents used in the chromatographic separation. Source: Pagnocca et al., 2006 [66].



(31)

Figure 13. Synthesis of amides 1-9. Source: Pagnocca et al., 2006 [66].

4. Control of leaf–cutting ants via enzymatic inhibition

When the cut plant fragments reach the colony, a complex process of preparation of the plant substrate for its incorporation into the fungal garden begins. During this processing, the workers may ingest the plant's sap while cutting and pressing the borders of the plant fragment. By scraping the surface, they remove the epicuticular wax layer and facilitate the decomposition of the substrate by the fungus [42,67] indicating the importance of the hydrolytic enzymes in this process. This behaviour is also related to the decontamination of the substrate [68].

Ultra-structural studies of the colonisation of the plant substrate by the fungus have demonstrated that the fungus can only use the portions that have had their border cut. In addition, the cuticular surface of the leaf at the time of colonisation appears to be intact, which suggests an absence of cutinases (enzymes that catalyse the hydrolysis of cutin, a structural component of the cuticle of the plant) in this process. Therefore, it is believed that this symbiotic fungus is a saprophyte that is unable to penetrate into plants that are not damaged [69] (Figure 13).

Therefore, maceration aids in the destruction of the physical barrier of the leaf cuticle, increasing permeability to allow fungal growth, which is assisted by enzymes present in the faecal fluid. [70] The symbiotic fungus is an important mediator involved in providing nutrition to the ants via the hydrolysis of polysaccharides from plant [71] as it produces large amount of enzymes, particularly pectinases, that are ingested by ants, concentrated in the intestine, returned to the fungal garden via faecal fluid, and utilised for the digestion of plant tissues [7]. Therefore, this association is also essential for fungal access to the nutrients in the plant material that is transported by the ants to the nest [71].

The profile of the hydrolytic enzymes involved in this relationship between leaf-cutting ants and fungi has been studied. The extracts from the fungal hyphae of garden fungi exhibit a wide range of activities involving carbohydratases (pectinase, laminarinase, α-glucosidase, β-glucosidase, α-galactosidase), with the proteinase chitinase presenting the highest activity (Erthal et al., 2009).

Extracts from fungal gardens contain a wide variety of digestive enzymes, including carbohydratases (e.g., pectinase, laminarinase, and β-1,3 glucanase) and proteinases (trypsinase and

chymotrypsinase) [69]. Interestingly, although the fungus (hyphal extracts) produces chitinase, the plants do not contain chitin. It has been suggested that the chitinases present in the rectal fluid of primitive genera (*Cyphomyrmex*, *Mycocepurus*, and *Myrmicocrysta*) are important in the degradation of substrates such as the carcasses of leaf-cutting ants, which can be used as a substrate for the growth of new hyphae from the symbiotic fungus. It has also been proposed that these enzymes may play an important role in the lysis of competitive funguses, many of which have cellular walls made of chitin [72].

The results from analysis of the labial glands of larvae and adults of *A. subterraneus* indicate that they secrete chitinases. Chromatographic tests to detect chitinolytic activity in the labial glands of the larvae revealed profiles similar to those observed for the glands of the workers, indicating that this enzymatic activity may have a fungal origin. Interestingly, the peak of chitinolytic activity in the middle intestine of the larvae does not coincide with the peak in the labial gland, indicating that the chitinase produced in the labial gland is not active in the middle intestine [73, 74].

In addition to chitinases, pectinases, xylanases, and cellulases derived from the fungus have been detected in the faecal fluid of *Atta colobica tonsipes* [75]. Pectinases present in the faeces of *Acromyrmex echinatior* and *Atta colombica* have been suggested to be of fungal origin [7]. Interestingly, no pectinase, xylanase, or cellulase activity was detected in *A. subterraneus* adults. However, elevated α-glycosidase activity was detected in fractions from the middle intestine and rectum of adult leaf-cutting ants, indicating the importance of this enzyme in the assimilation of glucose and, most likely, of saccharose and maltose present in the plant material [73]. Pectinases and xylanases were detected in the middle intestine of the larvae, where their only source of nutrients is the symbiotic fungus [74].

Febvay and Kermarrec (1984) suggested that digestion of the walls of the fungal cells occurs in the infrabuccal pocket of *Acromyrmex octospinosus*. It is possible that the adult garden ants regurgitate chitinases through their labial glands onto the fungal material to feed the larvae, initiating the partial digestion of fungal parts, without the adult benefiting from ingestion. The larvae can regurgitate their own secretions from their labial glands, providing the energetic needs of the adults, who receive these liquids through trophallaxis [76].

Other authors have reported that the secretions offered by the larvae originate in a liquid produced from the anal region, in a process referred to as proctodeal trophallaxis (Figure 14). The workers contact the anal region of the larvae, which may be of different sizes, and the larvae release a small transparent drop that is immediately ingested by the workers. This observation reveals that the larvae of the leaf-cutting ants play a fundamental role in the nutrient flux in the colony because, although adult individuals are incapable of feeding on the solid portions of the fungus, the larvae are able to feed on these portions, digest their walls, and transfer nutrients to the haemolymph, where they are absorbed by the Malpighian tubules, transferred to the posterior intestine, and offered to the workers, making them available to other individuals in the colony [77].

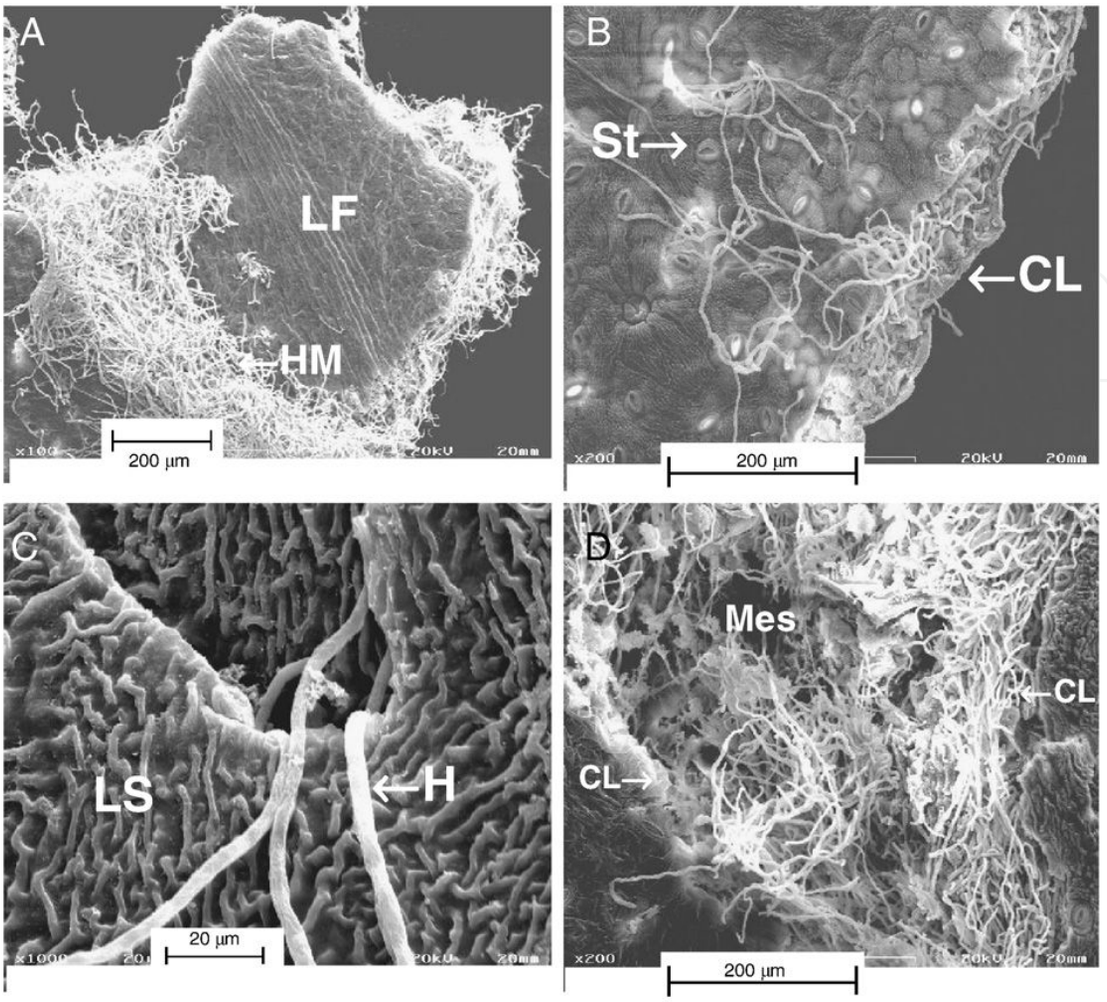


Figure 14. Colonisation of freshly cut leaves by *Leucoagaricus gongylophorus*. Panel A: LF—leaf fragment; HM—hyphal mass. Magnification: ×100. Panel B: St—stomata; CL—cut leaf edge exposing mesophyll tissue. Magnification: ×200. Panel C: H—hyphae; LS—leaf surface. Magnification: ×1000. Panel D: CL—cut leaf edge; MS—exposed mesophyll tissue. Magnification: ×200. Source: Erthal Jr. et al., 2009 [69].

Hydrolytic enzymes are directly involved in this energy transfer within the colony. Thus, studies that seek new insecticides have been conducted with an emphasis on plant extracts or pure substances that exhibit fungicidal, insecticidal, or enzyme inhibitory actions. The integrated application of these three types of functions should lead to the development of a new product with an effective control capacity. In this context, crude extracts of *Cedrela fissilis*, *Tapirira guianensis*, and *Simarouba versicolor* were evaluated and found to inhibit the activity of the pectinase enzyme present in the faecal liquid of *A. sexdens rubropilosa* [61,78]. These enzymes appear to be essential for the nutrition of the ants and the fungus in plant materials [71].

Plant extracts that inhibit enzymatic activity may be useful for the control of leaf-cutting ants and constitute a new approach with respect to methods for controlling these insects. This type of control should be evaluated further to determine the viability and effectiveness of its use in the field and confirm its suggested potential. Phytochemical analyses of active extracts are

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being conducted with the purpose of isolating the substances associated with enzymatic inhibition.

5. Conclusions

Data from the literature clearly demonstrate that several plants are capable of producing substances with direct action against leaf-cutting ants and/or their symbiotic fungi, such as ricinine (*Ricinus communis*; Euphorbiaceae); β-eudesmol (*Eucalyptus maculata*; Myrtaceae), the limonoid limonéxico acid (*Raulinoa echinata*; Rutaceae), sesamin and sesamoline (Sesamum indicum; Pedaliaceae), anthranilic acid, kokusaginine and dictamine (*Helietta puberula*; Rutaceae), 4.5- dimetoxicantin-6-one and 5-metoxicantin-6-one (*Simarouba versicolor*; Simaroubaceae), (-)-hinokinin and (-) kusunokin (*Virola sebifera;* Myristicaceae), among others. The active substances extracted from these plants may provide the basis for studies aimed at the synthesis of organic molecules and the development of new commercial products that are stable and show low persistence in the environment. In Brazil, these studies intensified after the establishment of restrictive policies by government entities and certifying institutions regarding the use of the active ingredients that are currently available in the market. Several molecules have already been synthesised, although they are not yet available for use by farmers; however, expectations for the use of plant-derived products in the control of leaf-cutting ants are high.

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