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PLANT EXTRACTS

Toxicity of Substances Isolated from *Helietta puberula* RE Fr. (Rutaceae) to the Leaf-cutting Ant *Atta sexdens* L. (Hymenoptera: Formicidae) and the Symbiotic Fungus *Leucoagaricus gongylophorus* (Singer) Möller

ROBERTA N. A. ALMEIDA[1], MARIA F. G. V. PEÑAFLOR[1], SIMONE Y. SIMOTE [2], ODAIR C. BUENO[1]; MARIA J. A. HEBLING[1], FERNANDO C. PAGNOCCA[1], JOÃO B. FERNANDES[2], PAULO C. VIEIRA[2], MARIA F. G. F. DA SILVA[2]

[1]*Centro de Estudos de Insetos Sociais, Universidade Estadual Paulista – UNESP, Caixa Postal 199, CEP 13506-900, Rio Claro, Brazil. E-mail: penaflor@esalq.usp.br*
[2]*Departamento de Química, Universidade Federal de São Carlos, Caixa Postal 676, CEP 13565-905, São Carlos-SP, Brazil.*

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Toxicidade de Substâncias Isoladas de *Helietta puberula* RE Fr. (Rutaceae) para as Formigas Cortadeiras *Atta sexdens* L. (Hymenoptera: Formicidae) e o Fungo Simbionte *Leucoagaricus gongylophorus* (Singer) Möller

RESUMO - As formigas cortadeiras são pragas que podem causar sérios prejuízos para a agricultura e silvicultura, uma vez que elas possuem o hábito de cortar material vegetal fresco para cultivar o fungo simbionte, sua principal fonte alimentar. Atualmente, métodos químicos têm sido largamente empregados para o controle de formigas cortadeiras e isso tem acarretado sérios problemas à saúde humana, assim como para o meio ambiente e outros organismos não-alvo. Nesse sentido, espécies vegetais são promissoras fontes de novas moléculas inseticidas que podem ser tóxicas tanto para as formigas cortadeiras quanto para o fungo simbionte, além de serem mais degradáveis e menos tóxicas para mamíferos. Em vista disso, o presente estudo objetivou a determinação da toxicidade de extratos, frações e substâncias puras de *Helietta puberula* RE Fr. (Rutaceae) para operárias médias de *Atta sexdens* L. (Hymenoptera: Formicidae) e para o seu fungo simbionte, *Leucoagaricus gongylophorus* (Singer) Möller. A toxicidade para as saúvas foi determinada através de bioensaios por ingestão, enquanto que com o fungo simbionte, foi avaliado o seu desenvolvimento em meio de cultura contendo os extratos vegetais. Os resultados mostraram que dentre as seis substâncias isoladas de *H. puberulla*, três delas foram concomitantemente tóxicas para as saúvas e para o fungo, mostrando ser essa espécie vegetal uma fonte promissora de novas substâncias para o controle de formigas cortadeiras.

PALAVRAS-CHAVE – extratos vegetais, atividade inseticida, atividade fungicida, Rutaceae, controle

ABSTRACT - Leaf-cutting ants may be a serious pest for the agriculture and silviculture once they cut plant material to cultivate a symbiotic fungus, which is their main food source. Currently, chemical methods are largely used for controlling of these insects, but sometimes with serious damages to human health, environment and other non-target organisms as well. Considering this, some plants are promising as a source of toxic substances, which can be toxic both to leaf-cutting ants and the symbiotic fungus. The main objective of this research was to determine the toxicity of extracts, fractions and isolated substances from *Helietta puberula* RE Fr. (Rutaceae) to *Atta sexdens* L. (Hymenoptera: Formicidae) workers and their symbiotic fungus, *Leucoagaricus gongylophorus* (Singer) Möller. Toxicity of vegetal extracts to leaf-cutting ants were determined by ingestion bioassays, while the activity against the symbiotic fungus was evaluated by its development in a culture medium containing vegetal extracts in it. The results showed that three out of six compounds isolated from *H. puberula* have effectively targeted both partners simultaneously, indicating that this plant is a promising source of novel insecticidal substances for the control of leaf-cutting ants.

KEYWORDS – vegetal extracts, insecticidal activity, fungicidal activity, Rutaceae, control

Leaf-cutting ants belonging to the genera *Atta* and *Acromyrmex* are widely distributed from Argentina to the Southern United State and they have been considered to be among the most destructive herbivores in this area (Della Lucia 1993). They cultivate a symbiotic fungus for feeding, using leaf fragments as substrate. As consequence of this behavior, they can cause serious damage to the agricultural crops (Cherrett & Justum 1983, Forti 1999), silviculture (Zanetti *et al*. 2003) and grazing land (Forti 1999).

Different methods have been proposed for the control of these ants, although the most used one is the chemical method. The chemical control can cause serious problems to human and non-target animals' health. In addition, the chemical insecticides are persistent, environmental contaminators agents and they can be incorporated to food chain. As a result, the use of this control strategy can cause ecological disequilibrium by selecting resistant populations (Lara & Batista 1992).

It is known that plants have several mechanisms to avoid herbivores, including a set of toxic substances (Harborne 1972). The presence of secondary plant metabolites which can be toxic to the ants and/or their symbiotic fungus plays an important role in host selection by the leaf-cutting ants (Hubbell *et al*. 1983). Thus, natural products of higher plants may represent a novel alternative method for controlling these economically important pests once they simultaneously target both leaf cutting ants and their symbiotic fungus (Bueno *et al.* 1990).

Some vegetal extracts have already been found to be potentially toxic not only to leaf-cutting ants or to symbiotic fungus, but also to both of them. Howard *et al.* (1988) verified that isolated terpenoids from *Hymenaea courbaril* L., *Melampodium divaricatum* (Rich.) DC. and *Vismia baccifera* (L.) showed toxic effects to *Atta cephalotes* L. (Hymenoptera: Formicidae) and to its symbiotic fungus. Like these terpenoids isolated from plants, other vegetal species also presented deleterious effects to leaf-cutting ants as well as to symbiotic fungus, for instance *Sesamum indicum* L. (Bueno *et al*. 1995, Ribeiro *et al*. 1998), *Ricinus communis* L. (Acácio-Bigi *et al.* 1998, Bigi *et al.* 2004), *Ipomoea batatas* (L.) (Hebling *et al.* 2000), *Canavalia ensiformis* (L.) DC (Monteiro *et al.* 1998, Takahashi-Del-Bianco 2002) and more recently for *Cedrela fissilis* Vell. (Bueno *et al.* 2005).

In a previous study, Pagnocca *et al.* (1996a) isolated lignins from *Virola sebifera* Aubl. which showed activity against *Atta sexdens* L. (Hymenoptera: Formicidae) and the symbiotic fungus. Among these lignins, the sesamine, inhibited fungus growth at high rates, and the value of this compound must be emphasized because of its wide range of occurrence in plants composition (Budowski 1964). The fungicide activity of *S. indicum*, for instance, must be due to the presence of sesamine in its extracts (Pagnocca *et al.* 1990, Pagnocca *et al*. 1996b). However, a more recent work showed that sesame seeds toxicity to *A. sexdens* workers is related to fractions containing triglycerides (Morini *et al.* 2005).

Regarding toxic substances for insect control, the Rutaceae family has been considered to be one of most important sources, since vegetal species belonging to it are great producer of secondary metabolites, which can vary from simple compounds to more complex ones, as limmonoids. Among the metabolites frequently found in this family, we can mention terpenoids, limmonoids, proto-limmonoids, coumarins, alkaloids and lignins (Paula *et al.* 1997), which can present insecticidal proprieties (Lewis 1983).

The aim of the present study was to determine the toxicity of crude extracts, methanolic fractions and substances isolated from stem, leaves and branches of *Helietta puberula* RE. Fr. to *A. sexdens* workers, by ingestion tests, and in the development of leaf-cutting ants' symbiotic fungus *Leucoagaricus gongylophorus* (Singer) Möller as well.

Material and Methods

Obtaining crude extracts and pure substances. The extracts have been prepared from different plant organs (stem, leaf and branch). Parts of plants were powdered, dried at 40ºC and percolated with a set of organic solvents (hexane, dicholoromethane and methanol) during 72 hours, three times at room temperature for three days, followed by evaporation of solvent under reduced pressure at 40ºC. The crude extracts were fracionated through fast chromatography under vacuity with silica gel and eluted with solvents of increasing polarity (hexane, dichlomethane, ethyl acetate and methanol). They were purified through different techniques including column chromatography, prepared plates and HPLC.

Ant bioassays. The worker ants *A. sexdens* used in the assays, whose body mass was about 20-25 mg, were from a laboratory nest kept at the Centro de Estudos de Insetos Sociais (Instituto de Biociências, Universidade Estadual Paulista- Rio Claro). Before the assays, the nests were supplied daily with leaves of *Eucalyptus alba* and occasionally with leaves of others plants such as *Hibiscus*.

Fifty ants were randomly picked up from the nest and put into 5 Petri dishes (ten ants each) for each treatment. During the assays the ants were maintained with a basic artificial diet (Bueno *et al.* 1997) which (control) had the following composition in g liter^{-1}: glucose (50); Bacto-peptone (10), yeast extract (1.0) and agar (15) in distilled water. The experimental diets were prepared by addition of the plant material (crude extract, partially purified extract or pure compound) to the basic formula. For a better distribution of the different plant material in the aqueous medium a mixture of dry constituents of the diet was prepared

(dry-mix), After the addition of water the material was autoclavated at 121°C /15 minutes, poured into Petri dishes, cooled and refrigerated. Blocks of 0.4 g per dish (control or experimental) were offered daily to the workers in a small peace of aluminum foil. The final concentrations of crude extracts, fractions and substances isolated from *H. puberula* in the diet were (mg mL^{-1}): 2.0; 1.6 and 0.3.

During the assays, the Petri dishes were maintained in an incubator at 25 (±1)°C and relative humidity between 70-80%. The maximum length of observation was 25 days and the number of dead ants was registered daily.

The survival median 50% (S_{50}) was calculated and compared by the computer-assisted software Prism™ 3.0 using the log-rank-test ($p<0.05$).

Fungus bioassays. The symbiotic fungus *L. gongylophorus* was isolated from a laboratory nest of *A. sexdens*. The medium for fungus maintenance and methods for bioassays were previously described (Pagnocca *et al.* 1990). Briefly, one ml of solvent (dichloromethane or methane) of each extracts and substances, were added to 9.0 ml of culture medium containing in g $liter^{-1}$: glucose (10); sodium chloride (5); peptone (5); malt extract (10) and agar (15). Control tubes received 1.0 ml of solvent and 9.0 ml of medium. After addition it was autoclavated at 121ºC for 15 minutes and slanted. The final concentration of the crude extracts, fractions and the molecules were ($mg.ml^{-1}$): 1.0, 0.2 and 0.05, respectively. The fungal suspension was prepared by transfering aseptically pieces of the mycelia (obtained from 1-month old culture growing in slant culture) to an all-glass tissue grinder containing sterile peptone ($1g.l^{-1}$) and weakly fragmented. One ml of this suspension was carefully spread on the surface of the agar slant and incubated at 25º (±1) C for 30 days. The assays were run twice (two sets of five tubes each). Fungal growth was estimated macroscopically on the basis of the mycelial surface and density after 30 days of incubation and the modal value was registered.

Results and Discussion

The data in Table 1 summarizes the percentage of fungal growth inhibition in presence of *H. puberula* crude extracts, fractions and isolated substances (Fig. 1). All crude extracts of *H. puberula* leaf and branch showed activity against the growth of symbiotic fungus. From these crude extracts, some substances that did not show any deleterious effect on fungus development were isolated, as flindersiamine and sitosterol, which were isolated from dichloromethane crude extracts of leaf and hexane crude extracts of branch, respectively. On the other hand, anthranilic acid, kokusaginine and maculine, also isolated compounds from these crude extracts, showed strong activity against the fungus growth, indicating they may be the main active compounds in the dichloromethane leaf and hexane branch crude extracts. From dichloromethane crude extract of branch, one active compound was also isolated, the dictamnine, which inhibited in 100% fungus development.

Biavatti *et al.* (2002) isolated some known furoquinoline alkaloids toxic to *L. gongylophorus* from stems and leaves extracts of the South Brazilian endemic plant *Raulinoa echinata* Cowan, Rutaceae: the widespread skimmianine; kokusaginine, maculine, flindersiamine, and also quinolone derivatives: 1-methyl-2-*n*-nonyl-4-quinolone, 2-*n*-nonyl-4-quinolone and 1-methyl-2-phenyl-4-quinolone. However, the results obtained for kokusaginine and maculine were distinct from those of the present work. The first inhibit in 100% and the last in only 50%. This divergence may be caused by the different fungus strains used in the assays.

Other alkaloids have been described as active substances against a wide range of microorganisms (Zhao *et al.* 1998, Rho *et al.* 1999) and has also been isolated from several rutaceous genera, mainly *Evodia* (Tang *et al.* 1996), *Boronia* (Sarker *et al.* 1995), *Ruta* (Grundon & Okely 1979) and *Esenbeckia* (Guilhon *et al.* 1994).

All methanol, hexane and dichloromethane crude extracts of *H. puberulla* leaves, stems and branches were significantly toxic to leaf-cutting ants (Table 1).The data in Tables 2 and 3 show, respectively, toxicity of methanol fractions and pure compounds of *H. puberula* to leaf-cutting ants workers (Table 4). Among methanol fractions, all dicloromethane and hexane were toxic to the ants. The purified substances anthranilic acid, kokusaginine and dictamnine were significantly toxic to leaf-cutting ant workers. However, a strong effect on ant mortality was not observed on treatments with purified compounds as occurred with extracts and fractions treatments. This loss of activity must have occurred during the different steps of purification, indicating that inhibitory activity could be attributable to the joint action of these compounds rather than to the action of a single substance (Bueno *et al.* 2005, Morini *et al.* 2005).

Other rutaceous plants, mainly the *Zanthoxylum* genus, that have great potential as source of toxic substances to herbivorous insects have already been described (Ogunwolu & Odumlami 1996, He *et al.* 2002) and to leaf-cutting ants, as *Citrus* sp. (Fernandes *et al.* 2002) and *R. echinata* (Biavatti *et al.* 2005).

Some authors suggested that toxicity of plants to both leaf-cutting ants and its symbiotic fungus can not be caused by necessarily the same active compounds (Bigi *et al.* 2004, Morini *et al.* 2005). However, *H. puberula* presented three purified compounds (anthranilic acid, kokusaginine and dictamnine) that were simultaneously toxic to leaf-cutting ants and the

symbiotic fungus. Only maculine was active exclusively against symbiotic fungus.

The high activity showed by *H. puberula* extracts, fractions and isolated substances against leaf-cutting ant as well as their symbiotic fungus suggest that it is a potential botanical insecticide that can be used to control this pest. The use of this plant also must have many advantages compared to chemical methods, as it must not pollute the environment.

In addition, the employment of extracts from *H. puberula* for controlling insects in the field might be also possible, as the main active compounds are alkaloids, which are regarded as stable substances (Chizzola *et al.* 2000).

Table 1. Inhibitory effect of crude extracts, fractions and molecules isolated of *H. puberula* against symbiotic fungus of leaf-cutting ant *A. sexdens.*

Organ	Crude extracts (1 mg.ml^{-1})	Fration (0.3 mg. ml^{-1})	Molecule (0.1 mg. ml^{-1})	% of inhibition
Stem	Hexane			20
	Dichloromethane			0
	Methanol			100
		Hexane		40
		Dichloromethane		20
		Ethyl Acetate		100
		Hidroalcoolic		40
Leaf	Hexane			100
	Dichloromethane			100
			Anthranilic Acid	80
			Flindersiamine	0
	Methanol			100
		Hexane		60
		Dichloromethane		60
		Ethyl Acetate		20
		Hidroalcoolic		20
	Hexane			100
Branch			Kokusaginine	80
			Maculine	100
			Sitosterol	0
	Dichloromethane			100
			Dictamnine	100
	Methanol			100
		Hexane		40
		Dichloromethane		20
		Ethyl Acetate		20
		Hidroalcoolic		20

Control with and without solvent: = 0% of inhibition. Dry weight of the fungal suspension: arithmetic mean = 6,6 ± 2,4 mg.mL^{-1}

Besides these isolated substances from *H. puberula*, there must be other biological substances in this plant derivated especially from methanol crude extracts that have not been investigated. Further research must be made focusing the identification of others biological active compounds from this almost chemically unknown plant and also its use in the field to control *A. sexdens*' nests.

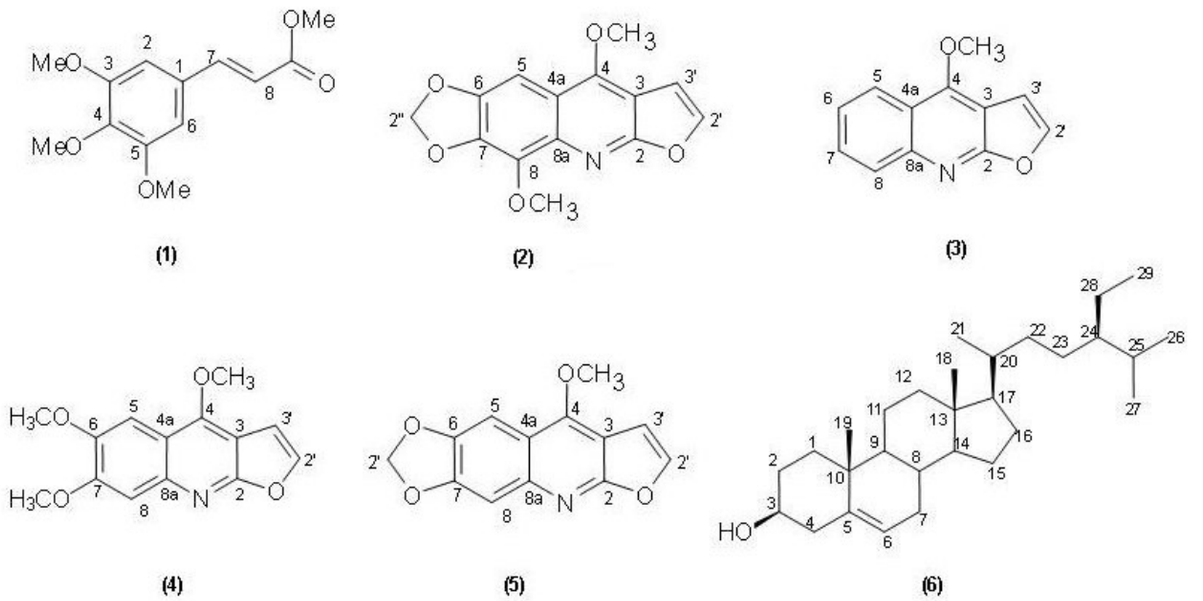

Figure 1. Molecular structures of substances isolated from *H. puberula,* (1) Anthranilic acid, (2) Flindersiamine, (3) Dictamnine, (4) Kokusaginine, (5) Maculine e (6) Sitosterol.

Table 2. Toxicity (% mortality and S_{50}) of *H. puberula* crude extracts to *A. sexdens* workers.

		Day of experiment										
Treatment²		1	2	3	6	8	10	14	17	21	25	S_{50}¹
Control		0	0	4	6	8	8	20	34	40	54	23 a
Leaf	D	0	2	16	50	60	66	70	72	82	82	6 b
	H	0	12	16	76	84	92	96	100	100	100	5 b
	M	0	0	8	50	60	72	78	84	90	90	8 b
Stem	D	0	0	6	62	74	84	86	86	90	92	6 b
	H	0	0	32	96	100	100	100	100	100	100	4 b
	M	0	0	0	14	22	30	48	54	66	78	16 b
Branch	D	0	0	0	14	22	34	38	52	66	76	17 b
	H	0	2	6	50	50	70	76	82	90	92	6,5 b
	M	0	6	30	64	66	66	68	76	88	92	5 b

¹S_{50}= survival median 50%. Different letters after the S_{50} values show a significant difference according to the log-rank test. ²D – Dichloromethane; H –Hexane; M – Methanol

Table 3. Toxicity (% mortality and S_{50}) of *H. puberula* methanol fractions to *A. sexdens* workers.

Treatment[2]		Day of experiment										
		1	2	3	6	8	10	14	17	21	25	S_{50}[1]
Control		0	2	2	8	18	24	36	50	56	68	17 a
Leaf	ME	0	0	0	2	12	24	48	70	80	88	15 a
	MH	0	2	6	38	56	70	88	90	96	100	7 b
	MD	0	0	2	18	36	44	74	78	82	90	11 b
	MHid	0	0	0	10	18	22	30	40	60	80	20 a
Stem	ME	2	4	4	12	18	26	48	64	78	84	15 a
	MH	0	0	2	12	30	54	76	90	94	94	10 b
	MD	0	0	2	22	34	44	68	84	92	94	13 b
	MHid	0	0	0	4	14	26	44	64	72	80	15 a
Branch	ME	0	0	2	4	6	10	32	54	66	76	17 a
	MH	0	2	4	20	34	58	76	86	92	92	9 b
	MD	0	0	0	10	32	46	66	88	90	92	11 b
	MHid	0	0	0	4	10	18	34	52	78	86	17 a

S_{50}= survival median 50%. Different letters after the S_{50} values show a significant difference according to the log-rank test. ME – methanol fraction of ethyl acetate; MH – methanolic fraction of hexane; MD – methanol fraction of dichloromethane; MHid - methanol fraction of hidroalcoolic

Table 4. Toxicity (% mortality and S_{50}) of *H. puberula* isolated substances to *A. sexdens* workers.

Treatment	Day of experiment										
	1	2	3	6	8	10	14	17	21	25	S_{50}[1]
Control	0	2	2	8	18	24	36	50	56	68	18 a
Sitosterol	0	0	0	6	6	10	30	46	62	80	20 a
Anthranilic Acid	0	0	0	2	10	24	56	72	74	86	14 b
Kokusaginine	0	0	0	6	10	22	42	58	74	90	17 b
Maculine	0	2	2	6	10	16	34	42	62	74	18 a
Flindersiamine	0	0	0	2	4	14	36	38	60	80	19 a
Dictamnine	0	0	0	2	6	14	52	62	84	88	14 b

S_{50}= survival median 50%. Different letters after the S_{50} values show a significant difference according to the log-rank test.

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