BioAssay

PLANT EXTRACTS

Toxicity of Substances Isolated from *Simarouba versicolor* St. Hil. (Simaroubaceae) to the Leaf-cutting Ant *Atta sexdens* L. (Hymenoptera: Formicidae) and the Symbiotic Fungus *Leucoagaricus gongylophorus* (Singer) Möller

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BioAssay 4:1 (2009)

Toxicidade de Substâncias Isoladas de *Simarouba versicolor* St. Hil. (Simaroubaceae) para Formigas Cortadeiras *Atta sexdens L.* (Hymenoptera: Formicidae) e para o seu Fungo Simbionte *Leucoagaricus gongylophorus* (Singer) Möller

RESUMO – O controle de formigas cortadeiras através da aplicação de inseticidas sintéticos pode ocasionar efeitos adversos ao meio ambiente, às populações de insetos não-alvo e à saúde do homem. Os produtos de origem vegetal, tóxicos aos insetos, representam uma alternativa viável de controle, uma vez que podem ser mais seletivos, além de apresentarem menor poder residual. Em vista disso, o presente estudo teve como objetivo verificar a toxicidade de extratos brutos, partições e substâncias isoladas de *Simarouba versicolor* St. Hil. (Simaroubaceae) para operárias de *Atta sexdens* L. (Hymenoptera: Formicidae) e seu fungo simbionte, *Leucoagaricus gongylophorus* (Singer) Möller. A toxicidade para as formigas foi determinada por meio de bioensaios por ingestão, enquanto que para o fungo simbionte, foi avaliado o desenvolvimento do mesmo em meio de cultura contendo os extratos vegetais. As frações diclorometânicas dos extratos metanólicos de *S. versicolor* mostraram atividade biológica para as formigas e para seu fungo simbionte. A partir dessas frações foram isolados dois alcalóides, 4,5-dimetóxicantin-6-ona e 5-metóxicantin-6-ona, sendo o primeiro tóxico somente para o fungo simbionte e outro para o fungo e a formiga. Os triterpenóides isolados de outros extratos de *S. versicolor* não mostraram efeito deletério para as formigas cortadeiras ou para o fungo simbionte.

PALAVRAS-CHAVE – Extratos vegetais, atividade inseticida, atividade fungicida, Simaroubaceae, controle.

ABSTRACT – Leaf-cutting ants' control by means of insecticide application can cause harmful effects to the environment, non-target insect populations and human health. Vegetal products toxic to insects may represent an alternative way for controlling economically important insects for the reason that they may be more selective, besides a less residual effect. In order of this, the aim of the current study was to evaluate the toxicity of crude extracts, fractions and isolated substances from *Simarouba versicolor* St. Hil. (Simaroubaceae) to *Atta sexdens* L. (Hymenoptera: Formicidae) workers and its symbiotic fungus, *Leucoagaricus gongylophorus* (Singer) Möller. Leaf-cutting ants' toxicity was determined by ingestion bioassays, while the activity against the symbiotic fungus was evaluated by its development in a culture medium containing vegetal extracts. Dichloromethane fractions derived from *S. versicolor* methanol crude extracts showed biological activity to leaf-cutting ants and its symbiotic fungus. From these fractions, two alkaloids were isolated, 4,5-dimethoxycanthin-6-one and 5-methoxycanthin-6-one, being the first toxic only to the symbiotic fungus and the other to fungus and the ant. Purified triterpenoids from others *S. versicolor* extracts didn't show deleterious effect to both leaf-cuttings ants and the symbiotic fungus.

KEYWORDS – Vegetal extracts, insecticidal activity, fungicidal activity, Simaroubacea, control.

Several studies have been carried out focusing the isolation of allelochemical compounds from toxic plants to economically important insects. This may be a way of obtaining new insecticidal molecules which use can avoid the harmful effects caused by the traditionally used pesticides in crops, like the decrease of non target insect populations and the appearance of resistant insects. Many important plant species that present substances which disrupt the insect behavior have been discovered in the past decades, such as, *Azadirachta indica* A. Juss and *Melia azedarach* L. and their biological active molecules have been also isolated (Isman 2002).

Leaf-cutting ants belonging to the genera *Atta* and *Acromyrmex* are considered among the most important pests of agricultural crops and man-made forests of exotic trees such as *Eucalyptus* spp. in Brazil (Zanetti *et al.* 2003). Traditional control of these ants with insecticides, in spite of its efficiency, is still a problem because of their non-selective toxicity (Vilela & Howse 1988). Therefore, it must be investigated other insecticidal molecules and ways of control these insects. Thus, the development of new insecticides from plant extracts sources have been proposed by many authors (Balandrin & Klocke 1985).

Bueno *et al.* (1990) suggested that several vegetal compounds may be toxic to leaf-cutting ants and/or the symbiotic fungus. Compounds that target both organisms may have good perspective in leaf-cutting ants' control. To date, vegetal species such as *Sesamum indicum* L. (Bueno *et al*. 1995, Ribeiro *et al*. 1998), *Virola sebifera* Aubl. (Pagnocca *et al.* 1996), *Ricinus communis* L. (Acácio-Bigi *et al.* 1998, Bigi *et al.* 2004), *Canavalia ensiformis* L. (DC) (Monteiro *et al.* 1999, Takahashi-Del-Bianco 2002), *Ipomoea batatas* (L.) (Hebling *et al.* 2000), *Cedrela fissilis* Vell. (Bueno *et al.* 2005) and, most recently, *Helietta puberula* RE Fr (Almeida *et al*. 2007) are known for deleterious effects to *Atta sexdens* L. and its symbiotic fungus at the same time, although biological active substances in the extracts for these two organisms can not be necessary the same.

Thus, the aim of the current study was to determine the toxicity of crude extracts, methanolic fractions and purified substances derived from stem, leaves and branches of *Simarouba versicolor* St. Hill. (Simaroubaceae) to *Atta sexdens* L. (Hymenoptera: Formicidae) workers, by ingestion tests, and in the development of leaf-cutting ants' symbiotic fungus *Leucoagaricus gongylophorus* (Singer) Möller as well.

Material and Methods

Obtainment of the crude extracts and pure substances. The extracts have been prepared from *S. versicolor* organs (stem, leaf and branch). Parts of plants were powdered, dried at 40°C 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 40°C. 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). The substances (4,5-dimethoxycanthin-6-one; 5-methoxycanthin-6-one; lupeol; and lupenone) were purified through different techniques including column chromatography, prepared plates and HPLC.

Ants' 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* sp.

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 $g.l^{-1}$: 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, fractions or isolated substances) 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 *S. versicolor* in the diet were ($mg.ml^{-1}$): 2.0; 1.6 and 0.3.

During the assays, the Petri dishes were maintained in a climate chamber 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.

To assess the effects of the treatments, percentage survivals were plotted as a function of time in a survival curve which was then used to calculate the survival median 50% (S_{50}). The survival curves were 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 rubropilosa*. The medium for fungus maintenance and methods for bioassays were previously described (Pagnocca *et al*. 1990). Briefly, 1 ml of solvent (dichloromethane or methane) of each extracts and substances, was added to 9 ml of culture medium containing in $g.l^{-1}$: glucose (10), sodium chloride (5), peptone (5), malt extract (10) and agar (15). Control tubes received 1 ml of solvent and 9.0 ml of medium.

After addition it was autoclavated at 121°C for 15 minutes and slanted. The final concentration of *S. versicolor* crude extracts, fractions and the molecules were: 1.0, 0.2 and 0.05 mg.ml^{-1}, respectively. The fungal suspension was prepared by transferring aseptically pieces of the mycelia (obtained from 1-month old culture growing in slant culture) to an all-glass tissue grinder containing sterile peptone (1 g.l^{-1}) and weakly fragmented. One ml of this suspension was carefully spread on the surface of the agar slant and incubated at 25±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 and 2 summarize the percentage of fungal growth inhibition in presence of *S. versicolor* crude extracts, fractions and isolated substances. All methanol crude extracts were highly toxic to the symbiotic fungus while only dichloromethane crude extracts from leaves and branches showed similar toxicity. By contrast, none of hexane crude extracts inhibited the fungus development in more than 20%, which is a weak activity.

Among the methanol fractions, all dichloromethane were significantly deleterious to the fungus, besides hydroalcoholic from stem and branches and ethyl acetate from leaves and branches. On the other hand, fractions extracted with hexane didn't show a strong activity against the symbiotic fungus, except the one from branches that was moderately toxic to the fungus (60% of inhibition).

The triterpenoids isolated from the hexane methanol fraction of *S. versicolor* stem and dichloromethane crude extract of branches, lupenone and lupeol, respectively, showed none or just a slight (20%) inhibitory effect to the symbiotic fungus (Table 2). Although, alkaloids (5-methoxycanthin-6-one and 4,5-dimethoxycanthin-6-one), isolated from dichloromethane methanol fraction of *S. versicolor* stem and branches, respectively, inhibited in 100% the fungus' development (See Fig. 1 for chemical structure of the isolated substances).

The data in Table 3 and 4 show toxic effects of crude extracts, methanol fractions and isolated substances of *S. versicolor* to leaf-cutting ants. All methanol and dichloromethane crude extracts from *S. versicolor* leaves, stem and branches were significantly toxic to leaf-cutting ants. Only one hexane crude extract (branches) showed some toxic effect to *A. sexdens* workers.

Among methanol fractions, all dichloromethane were highly toxic to leaf-cutting ants workers, decreasing the survival median from 16 days (control) to only approximately 4 (stem and leaves) and 3 days (branches). Deleterious effects to *A. sexdens* workers of treatments with ethyl acetate from leaves and branches and hydroalcoholic from branches were also observed.

Table 1. Inhibitory effect of *Simarouba versicolor* crude extracts and methanol fractions against symbiotic fungus of leaf-cutting ant *Atta sexdens.*

Plant Part	C/F[1]	Solvent	% of inhibition[2]
Stem	C	Hexane	0
	C	Dichloromethane	40
	C	Methanol	80
	F	Hexane	20
	F	Dichloromethane	100
	F	Ethyl Acetate	40
	F	Hydroalcoholic	100
Leaves	C	Hexane	20
	C	Dichloromethane	100
	C	Methanol	100
	F	Hexane	0
	F	Dichloromethane	80
	F	Ethyl Acetate	100
	F	Hydroalcoholic	0
Branches	C	Hexane	0
	C	Dichloromethane	100
	C	Methanol	100
	F	Hexane	60
	F	Dichloromethane	100
	F	Ethyl Acetate	100
	F	Hydroalcoholic	100

[1]C = crude extract (concentration of 1.0 mg.ml^{-1}); F = fraction (concentration of 0.3 mg.ml^{-1});
[2]Control with and without solvent: = 0% of inhibition; Dry weight of the fungal suspension: arithmetic mean = 6.6 ± 2.4 mg.mL^{-1}.

Alkaloids derived from plants may play a defensive role against microorganisms, insects and mammal herbivores (Levin 1976). Furthermore, these compounds can also act as fungistatic, as described by Michael (2000) and Singh *et al.* (2000).

Regarding the toxicity to leaf-cutting ants, alkaloids have been extensively regarded as promising molecules for controlling. Several works demonstrated the high deleterious effects of alkaloids as the main responsible substances of biological activity of plant extracts (Bigi *et al.* 2004, Almeida *et al.* 2007).

Table 2. Inhibitory effect of *Simarouba versicolor* isolated substances against symbiotic fungus of leaf-cutting ant *Atta sexdens.*

Molecule[1]	Isolated from	% of Inhibition[2]
Lupenone	Hexane methanol fraction from stem	0
5-methoxycanthin-6-one	Dichloromethane methanol fraction from stem	100
Lupeol	Dichloromethane crude extract from branches	20
4,5-dimethoxycanthin-6-one	Dichloromethane methanol fraction from branches	100

[1]Substances at concentration of 0.1 mg.ml^{-1};
[2]Control with and without solvent: = 0% of inhibition; Dry weight of the fungal suspension: arithmetic mean = 6.6±2.4 mg.mL^{-1}.

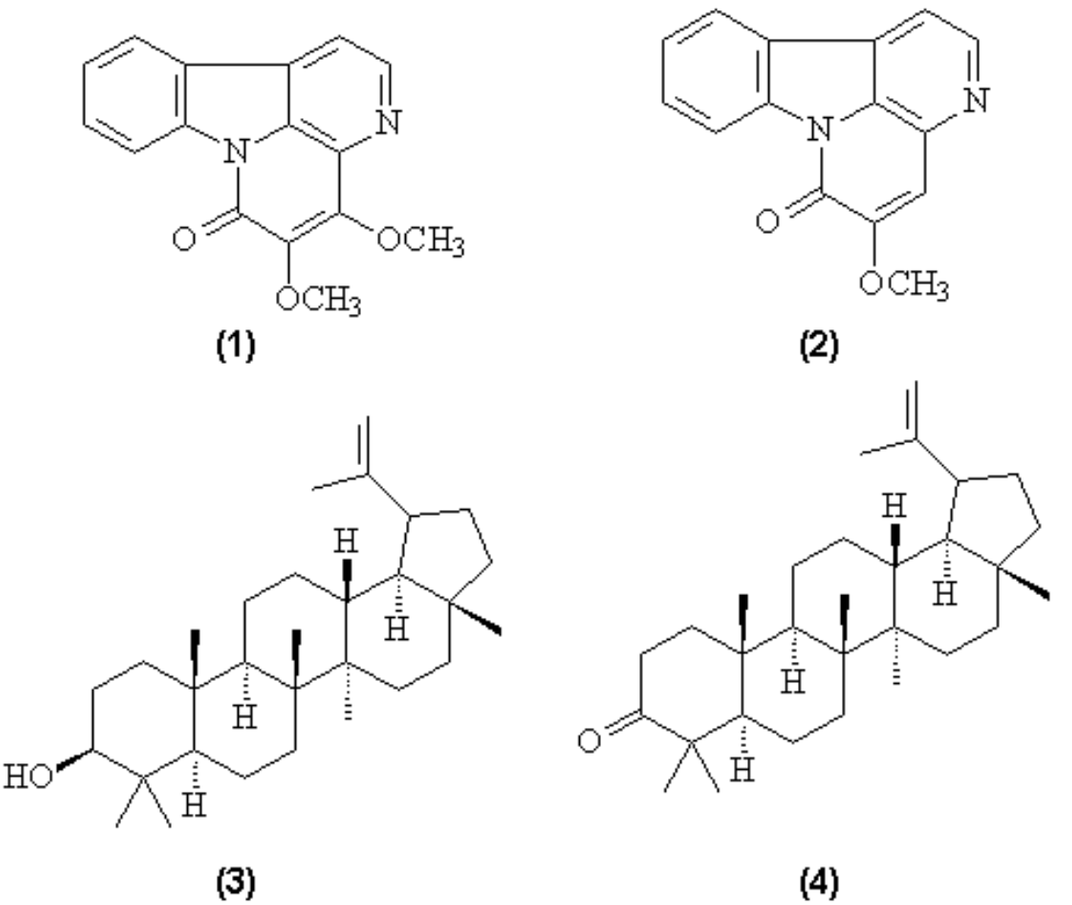

Figure 1. Chemical structures of substances isolated from *S. versicolor*: (1) 4,5-dimethoxycanthin-6-one; (2) 5-methoxycanthin-6-one; (3) lupeol; (4) lupenone.

Table 3. Toxicity (% mortality and S_{50}) of *S. versicolor* crude extracts to *Atta sexdens* workers.

Plant part	C/F[1]	Solvent	Day of experiment										
			1	2	3	6	8	10	14	17	21	25	S_{50}[2]
Control			0	0	0	2	8	14	36	52	66	82	16 a
Leaves	C	Hexane	0	0	0	8	10	24	46	64	72	78	16 a
	C	Dichloromethane	0	2	2	20	36	60	86	92	94	98	10 b
	C	Methanol	0	2	10	48	68	74	96	96	100	100	7 b
	F	Ethyl Acetate	0	0	0	20	42	50	66	78	86	88	9 b
	F	Hexane	0	0	4	6	16	24	44	54	70	74	16 a
	F	Dichloromethane	0	4	30	76	82	86	100	100	100	100	4 b
	F	Hydroalcoholic	0	0	0	0	8	14	36	48	76	84	18 a
Stem	C	Hexane	0	0	0	0	8	16	44	70	84	86	15 a
	C	Dichloromethane	0	0	2	26	44	64	80	84	86	90	9 b
	C	Methanol	0	0	6	58	82	86	88	90	96	100	6 b
	F	Ethyl Acetate	2	12	36	82	86	92	96	96	100	100	4 b
	F	Hexane	0	2	6	32	42	50	70	78	84	90	10 a
	F	Dichloromethane	2	22	44	82	86	94	96	100	100	100	4 b
	F	Hydroalcoholic	2	8	12	34	48	56	68	76	86	86	10 a
Branches	C	Hexane	2	6	6	10	20	26	56	76	86	88	14 b
	C	Dichloromethane	0	0	4	40	74	82	96	98	98	98	7 b
	C	Methanol	0	2	12	36	56	70	80	86	88	88	8 b
	F	Ethyl Acetate	0	0	8	44	62	72	82	86	90	90	7 b
	F	Hexane	4	10	18	32	42	46	60	68	80	88	11 a
	F	Dichloromethane	4	30	58	86	98	98	100	100	100	100	3 b
	F	Hydroalcoholic	0	8	12	28	46	60	78	86	92	92	9 b

[1]C = crude extract (concentration of 2.0 mg.ml^{-1}); F = fraction (concentration of 1.6 mg.ml^{-1});
[2]S_{50}- survival median 50%. Different letters after the S_{50} values show a significant difference according to the log-rank test. Different letters after the S_{50} values from its respective control mean significant difference according to the log-rank test ($P<0.05$).

Table 4. Toxicity (% mortality and S_{50}) of *S. versicolor* isolated substances to *Atta sexdens* workers.

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	14 a
Lupenone	4	4	6	12	24	30	46	54	64	76	16 a
4,5-dimethoxycanthin-6-one	2	2	8	34	36	48	56	58	68	82	13 a
Lupeol	2	2	10	26	26	28	34	36	44	54	19 a
5-methoxycanthin-6-one	0	0	10	46	52	56	76	78	80	88	7 b

[1]Isolated substances at concentration of 0.3 mg.ml^{-1};
[2]S_{50} = survival median 50%. Different letters after the S_{50} values show a significant difference according to the log-rank test. Different letters after the S_{50} values from its respective control mean significant difference according to the log-rank test ($P<0.05$).

Quassinoids have been reported as being the most important secondary metabolites from plants of Simaroubaceae family as they are regarded as the main chemotaxonomics markers (Da Silva & Goltlieb 1987). Moreover, these substances are responsible for insecticidal effects, disrupting insect feeding behavior or the metamorphosis provided by some species of Simaroubaceae (Latif *et al.* 2000, Govindachari *et al.* 2001).

Particularly *S. versicolor* has as constituents quassionoids, triterpenoids, a mixture of steroids, a flavonoid and a squalene derivative from roots, stems and fruits (Arriaga *et al*. 2002). Among these, it has already been described the biological activity of flavonoids (Cintra *et al.* 2005) and terpenoids (Howard *et al.* 1988, Marinho *et al.* 2005) against leaf-cutting ants.

Recently, Coelho *et al.* 2006 showed a high toxic effect of *S. versicolor* roots to *Rhodnius milesi* Carcavallo, Rocha, Galvão & Jurberg (Hemiptera: Reduviidae), but these authors didn't isolate active substances from the extracts.

Rutaceous plants are known as a great potential source of toxic substances to herbivorous insects (Ogunwolu & Odumlami 1996, He *et al.* 2002) and, specifically, to leaf-cutting ants, like *Citrus* sp. (Fernandes *et al*. 2002) and *Raulinoa echinata* Cowan (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, *S. versicolor* presented one substance, the alkaloid 5-methoxycanthin-6-one, which was simultaneously toxic to leaf-cutting ants and the symbiotic fungus. The other isolated alkaloid (4,5-dimethoxycanthin-6-one) showed activity exclusively against symbiotic fungus.

Besides these isolated substances from *S. versicolor*, there must be other biological active substances in this plant derivated from dichloromethane crude extracts that have not been investigated.

The current study showed that *S. versicolor* has potential as a source of active substances to control leaf-cutting ants' nests, because they target both organisms of the symbiotic relationship. In order to that, the control of leaf-cutting ants can be more effective and the harmful effects provided by the use of traditional insecticides can also be avoided. As the active substance for the leaf-cutting ants and its symbiotic fungus is an alkaloid (5-methoxycanthin-6-one), the employment of it on the field must be possible because of its stability.

Further research must be made focusing the identification of others biological active compounds from this promising insecticidal plant. Furthermore, field tests using isolated active substances for controlling the leaf-cutting ants' nest must be carried out to evaluate their effectiveness.

Acknowledgment

The authors are grateful to Carlos Ribeiro for reviewing the language, FAPESP (Fundação de Amparo a Pesquisa do Estado de São Paulo), CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico) and PRONEX-MCT/FINEP for financial support for this research as well as for the scholarship.

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Received: 18/X/2008 Accepted: 02/XI/2008 Published: 22/VI/2009 Available online: www.bioassay.org.br/ojs/index.php/bioassay/article/view/24/65