

Bioactivity of Flavonoids Isolated from *Lychnophora markgravii* against *Leishmania amazonensis* Amastigotes

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The bioactivity of the flavonoids pinostrobin (**1**), pinocembrin (**2**), tectochrysin (**3**), galangin 3-methyl ether (**4**), and tiliroside (**5**) isolated from *Lychnophora markgravii* aerial parts was investigated *in vitro* against amastigote stages of *Leishmania amazonensis*. The compounds were isolated by several chromatographic techniques and their chemical structures were established by ESI-MS and NMR spectroscopic data. The flavonoids **1** and **3** were the most active compounds; they markedly reduced the viability of *Leishmania* amastigotes.

Key words: *Lychnophora markgravii*, Flavonoids, Biological Activity

Introduction

The genus *Lychnophora* (Asteraceae) comprises approximately 34 species that are restricted to the Brazilian “Cerrado” (Coile and Jones, 1981; Robinson, 1999) and some species are known in Brazilian folk medicine as “arnica da serra”, “arnica brasileira” or “falsa arnica”. Extracts from *Lychnophora* plants are used in Brazilian traditional medicine for the treatment of infectious and inflammatory diseases and several studies reported anti-inflammatory and analgesic properties for alcoholic and hydroalcoholic preparations of aerial parts of *Lychnophora* (Gobbo-Neto and Lopes, 2008; Santos *et al.*, 2005). Moreover, studies documenting antioxidant, antimicrobial and trypanocidal properties of *Lychnophora* extracts indicated that species of this genus are a source of active agents (Takeara *et al.*, 2003; Graef *et al.*, 2000). Previous phytochemical investigations of extracts of *Lychnophora* sp. yielded sesquiterpene

lactones, lignans, flavonoids and caffeoylquinic acid derivatives (Gobbo-Neto and Lopes, 2008; Sakamoto *et al.*, 2003; Takeara *et al.*, 2003; Sartori *et al.*, 2002; Graef *et al.*, 2000).

Lychnophora markgravii G. M. Barroso is a Brazilian endemic plant restricted to Grão-Mongol and Cabral Hills of Minas Gerais, Brazil. Chemical analyses have demonstrated the occurrence of steroids, triterpenes and sesquiterpene lactones in *L. markgravii* roots extracts, while flavonoids were detected in aerial parts of this plant (Sartori *et al.*, 2002). No biological activity evaluation has previously been reported for *L. markgravii*.

Preliminary evaluations revealed leishmanicidal properties for dichloromethanic and ethanolic extracts of *L. markgravii*, and this prompted us to identify active constituents in these extracts. The bioassay-guided study on *L. markgravii* yielded five flavonoids with leishmanicidal activity *in vitro* against *Leishmania amazonensis* amastigotes.

Experimental

General experimental procedures

The IR spectra were obtained with KBr pellets using a Perkin Elmer model 1420 spectrophotometer. ^1H NMR (300 MHz) and ^{13}C NMR (75 MHz) spectra were recorded on a Bruker Avance DPX 300 instrument, in $\text{MeOH}-d_6$ or $\text{DMSO}-d_6$ with TMS as internal standard. ESI-MS was conducted on a Micromass Quattro-LC instrument. The UV spectra were obtained using a Hitachi U-3501 spectrophotometer. HPLC analysis was carried out on an LC-6A Shimadzu liquid chromatograph equipped with a 3501 UV detector and using a reverse phase separation procedure. The columns ODS-Shimpack (C-18, 4.6×250 mm, $5 \mu\text{m}$) and Shim-pack ODS (C-18, 20×250 mm, $5 \mu\text{m}$) were used for analytical and preparative procedures, respectively. TLC was carried out on Si gel PF-254 (Merck, Darmstadt, Germany), and CC on Si gel 60 (Merck) and Sephadex LH-20 (Sigma, St. Louis, MO, USA). In the biological assay, MTT cleavage was measured by using a multiwell scanning spectrophotometer (model #3550; Bio-rad Laboratories, Richmond, CA, USA) with the reference wavelength 655 nm and test wavelength 595 nm.

Plant material

Lychnophora markgravii G. M. Barroso (Asteraceae) aerial parts were collected in April 1996, by Dr. Walter Vichnewski, near the town of Grão-Mongol, MG, Brazil. The identification was performed by Dr. João Semir, Departamento de Botânica, Instituto de Biologia, UNICAMP, SP, Brazil, where a voucher specimen has been deposited (UEC35.144, Vichnewski collection # 402).

Extraction and isolation of compounds

The powdered, air-dried aerial parts of *L. markgravii* (750 g) were extracted exhaustively by maceration at room temperature with dichloromethane (CH_2Cl_2) and ethanol successively. The spent biomass was separated from the extracts by filtration, and the solvents were removed under reduced pressure in a rotatory evaporator (below 40°C), to obtain the dichloromethanic (80 g) and ethanolic (40 g) crude extracts.

The dichloromethanic extract (50 g) was suspended in $\text{MeOH}/\text{H}_2\text{O}$ (9:1), filtered and partitioned with hexane and CH_2Cl_2 . The hexane

portion (3 g) was recrystallized (diethyl ether/petroleum ether, 2:1), affording 650 mg of pinostrobin (**1**). The dichloromethane portion (15 g) was chromatographed over silica gel 60H (VLC) and 11 fractions were collected (10 mL each). Fraction II (hexane/EtOAc, 9:1) was rechromatographed by HPLC analysis (preparative ODS column; 9 mL/min; 286 nm; $\text{MeOH}/\text{H}_2\text{O}$, 7:3), furnishing 5 mg of pinocembrin (**2**) and 15 mg of tectochrysin (**3**). Fraction V (hexane/EtOAc, 7:3) was rechromatographed by HPLC analysis (preparative ODS column; 9 mL/min; 284 nm; MeOH/THF , 5.5:4.5), affording 25 mg of galangin 3-methyl ether (**4**).

An 1-g aliquot of the ethanolic extract was applied to a Sephadex LH-20 (Sigma, 400 g) column ($80 \text{ cm} \times 5.0 \text{ cm}$) using distilled MeOH as eluent to afford 36 fractions (12 mL each; 2.4 mL/min). These fractions were analyzed by TLC on silica plates using *n*-BuOH/acetic acid/ H_2O (65:10:25) as developing solvent. The combined fraction VI furnished 125 mg of tiliroside (**5**).

Bioassay

L. amazonensis (strain designation MPRO/BR/72/M 1841) amastigotes were maintained and cultured axenically according to established protocols (Pral *et al.*, 2003; Salvador *et al.*, 2002). Washed parasites were resuspended in RPMI-1640 medium supplemented with 4% fetal calf serum, pH 5.0, and incubated at 33°C for 18 h with crude extracts (1 mg/mL) or isolated compounds (0.014, 0.084, 0.5, and 1.0 mg/mL) dissolved in dimethyl sulfoxide (DMSO)/RPMI-1640 (1:99). The amastigote viability was assessed colorimetrically by the reduction of a tetrazolium salt (MTT) as described by Mosmann (1983). Absorbances were expressed as percentages relative to untreated controls. Amphotericin B (0.02 mg/mL) was used as the positive control and DMSO/RPMI-1640 (1:99) as negative control. The bioassays were carried out in triplicate.

Statistical analysis

Data are reported as means (%RSD, relative standard deviation) of triplicate determinations. The statistical analyses were carried out using the Microsoft Excel 2002 software package (Microsoft Corp., Redmond, WA, USA).

Results and Discussion

The dichloromethanic and ethanolic extracts from *L. markgravii* aerial parts were initially screened for their toxicity against axenic *L. amazonensis* amastigotes. Both extracts showed capacity to interfere markedly with the viability of *L. amazonensis* amastigotes: at a dose of 1 mg/mL, the parasite viability was reduced to 3.5 and 3.8%, respectively. Bioassay-guided fractionation of *L. markgravii* was carried out and five different compounds were purified by several chromatographic techniques. By comparing their physical and spectroscopic properties, including NMR (^{13}C , DEPT and ^1H), ESI-MS, IR and UV spectra, with those reported in the literature (Harborne, 1996; Markam and Geiger, 1994; Agrawal, 1989), the purified compounds (indicated in Fig. 1) were identified as pinostrobin (**1**), pinocembrin (**2**), tectochrysin (**3**), galangin 3-methyl ether (**4**), and tiliroside (**5**). The compounds **1**, **2**, **3**, and **4** were obtained from the dichloromethanic extract and the ethanolic extract furnished compound **5**.

Results of the bioassays with *L. amazonensis* amastigotes indicated the purified flavonoids **1** and **3** as the most active compounds (Table I). In the latter case, parasite viability was reduced to 11.9 and 15.4%, respectively, when the compounds were assayed at 0.5 mg/mL. These results plus

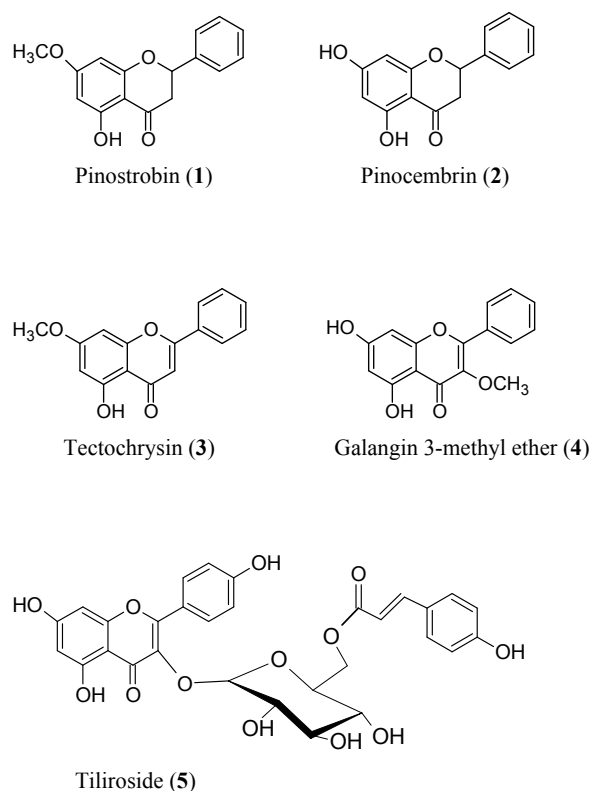


Fig. 1. Chemical structures of the antimicrobial compounds isolated from *Lychnophora markgravii*.

Table I. Effect of flavonoids **1–5** isolated from *Lychnophora markgravii* aerial parts on the viability of *Leishmania amazonensis* amastigotes.

Compound	Viability of <i>Leishmania amazonensis</i> amastigotes (CV) ^a (%)				IC ₅₀ [μM]
	0.014 mg/mL	0.084 mg/mL	0.5 mg/mL	1.0 mg/mL	
1	95.4 (2.3)	49.6 (4.5)	11.9 (5.9)	2.1 (3.0)	0.31
2	100.0 (5.6)	92.0 (4.5)	82.2 (4.2)	36.6 (2.6)	3.45
3	96.2 (2.6)	51.8 (3.5)	15.4 (2.8)	3.6 (2.0)	0.56
4	100.0 (10.3)	98.0 (8.8)	87.6 (5.3)	32.5 (4.2)	2.89
5	100.0 (2.2)	100.0 (7.9)	67.8 (4.6)	30.3 (5.1)	1.13
Amphotericin B (0.02 mg/mL) ^b	2.3 (9.1)				–
DMSO/RPMI-1640 (1:99) ^c	100.0 (4.5)				–

All experiments were run in triplicate.

^a Parasite viability was determined by the MTT reduction assay. O.D. values are expressed as percentage of those of untreated (negative) controls. CV = coefficient of variation (%) = $(s/m) \cdot 100$, where s is the standard deviation and m the mean. The IC₅₀ (concentration of compound that reduced response by 50%) value was estimated by graphical interpolation.

^b Positive control.

^c Negative control.

– Not evaluated.

those already reported in the literature (Taleb-Contini *et al.*, 2004) suggest that there may be a relationship between the leishmanicidal activity and the number and arrangement of the hydroxy groups of the flavonoids.

Amphotericin B (0.02 mg/mL), here used as a positive control, reduced the amastigote viability to 2.3%. *L. amazonensis* amastigotes incubated in medium alone or in medium containing DMSO/RPMI-1640 (1:99) remained viable during the 18-h incubation period (% amastigote viability = 100%), a result that excludes spontaneous parasite damage during the bioassay (Salvador *et al.*, 2002).

The results documented here indicate that the isolated flavonoids from *L. markgravii* display leishmanicidal activity *in vitro*; their bioactivity is evaluated for the first time. However, further investigations (*in vitro* and *in vivo*) are necessary to confirm the leishmanicidal potential of this plant and its constituents looking toward a clinical employment.

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