SUPPLEMENTARY MATERIAL

Antibacterial and Antifungal Properties Of Crude Extracts and Isolated Compounds From *Lychnophora Markgraviia*

Alvaro José Hernández-Tasco, Román Yesid Ramírez-Rueda, Carlos José Alvarez, Fabiana Terezinha Sartori, Ana Claudia B.C. Sacilotto, Izabel Yoko Ito, Walter Vichnewski, Marcos José Salvador

*Plant biology department, State University of Campinas (UNICAMP), Brazil; Chemistry department, University of São Paulo (USP), Brazil; Departments of Physics and Chemistry, and Clinical, Toxicological and Bromatological Analysis, University of São Paulo (USP), Brazil; Microbiology department, University of Pamplona, Colombia; † In memory.*

Abstract

Antimicrobial activity of dichloromethane and ethanol extracts and five compounds: pinostrobin (I), pinocembrin (II), tectochrysin (III), galangin 3-methyl ether (IV) and tiliroside (V) isolated from *Lychnophora markgraviia* aerial parts against fifteen microorganisms was determined. The structures of these compounds were elucidated based on ESI-MS and NMR spectroscopic data. Both extracts showed antimicrobial activity against several tested microorganisms. Pinostrobin, tectochrysin and galangin 3-methyl ether showed the strongest antibacterial and antifungal effects.

**Keywords:** *Lychnophora markgraviia*, flavonoids, antimicrobial activity, plant extract.

Experimental

General Experimental Procedures

The leaves of *L. markgraviia* was used to obtain extracts of dichloromethane and ethanol, in addition, 5 compounds were isolated by chromatographic fractionation on silica gel 60H, ODS and Sephadex LH-20 and determined by $^1$H NMR (300 MHz) and $^{13}$C NMR (75 MHz) spectra and ESI-MS/MS-MS. These extracts and isolated compounds were
challenge with strains of bacteria and fungi through the agar diffusion and microdilution
tests to determinate its antimicrobial properties.

**Plant material**

*Lychnophora markgravii* G. M. Barroso (Asteraceae), aerial parts were collected at the
rural area of Grão-Mongol, (MG, Brazil). The identification was performed by Dr.
Semir, from UNICAMP Institute of Biology (SP, Brazil), where a voucher specimen
was deposited (UEC35.144, Vichnewski collection # 402).

**Obtention of crude extract and fractions**
The extracts of air-dried aerial parts of *L. markgravii* (750 g) were obtained by
maceration at room temperature with dichloromethane (AR) and ethanol (AR)
successively. 50 g of dichloromethane extract was suspended in MeOH/H$_2$O (9:1),
filtered and partitioned with hexane and dichloromethane. The hexane portion (3 g) was
recrystallized (diethyl ether/petroleum ether, 2:1), affording 650 mg of pinostrabin (I).
The dichloromethane portion (15 g) was chromatographed over silica gel 60H (Vacuum
liquid chromatography) and 11 fractions were collected (10 mL each). Fraction II
(hexane/EtOAc, 9:1) was re-chromatographed by HPLC (preparative ODS column, 9
mL/min, 286 nm, MeOH/H$_2$O, 7:3), furnishing 5 mg of pinocembrin (II) and 15 mg of
tectochrysin (III). Fraction V (hexane/EtOAc, 7:3) was re-chromatographed 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 (IV). Ethanol extract (1 g) was applied to a Sephadex
LH-20 (Sigma, 400 g) column (80 cm x 5.0 cm) using distilled MeOH as eluent to
afford 36 fractions (12 mL each, flow rate 2.4 mL/min). These fractions were analyzed
by thin layer chromatography (TLC) on silica plates using n-BuOH-acid acetic- H$_2$O
(65:15:25) as developing solvent. The combined fraction VI furnished 125 mg of
tiliroside (V). The structures of all compounds were determined by $^1$H NMR (300 MHz)
and $^{13}$C NMR (75 MHz) spectra, which were measured in MeOH-d$_6$ or DMSO-d$_6$
using tetramethylsilane as the internal standard by a BRUCKER DPX 300 instrument
(400 MHz for $^1$H and 100 MHz for $^{13}$C). ESI-MS/MS-MS was measured on a
Micromass Quattro-LC instrument and on a mass spectrometer (Acquity TQD mass
spectrometer, Micromass Waters, Milford, MA, USA). The UV spectra were obtained
by Hitachi U-3501 spectrophotometer.
Antimicrobial activity

Crude extracts and isolated compounds were tested for antimicrobial activity against 15 strains of microorganisms including gram-positive, gram-negative bacteria and yeasts from ATCC and FCFRP/USP collections. (Table 1). Tests were performed by a modified agar-well diffusion method (well technique in double layer) using Mueller Hinton Agar for non-fastidious microorganisms and Brain Heart Infusion Agar for fastidious bacteria (Salvador et al. 2002; Ferreira et al. 2004). The used inoculum size was $10^6$ CFU/mL and the culture medium for antimicrobial test were Mueller Hinton Agar (MHA) for non-fastidious microorganisms and Brain Heart Infusion Agar (BHIA) for Streptococcus strains. Subsequently, aliquots of 20 μL of each test-sample solution were applied into 5.0 mm diameter wells. Solutions were prepared in dimethylformamide (DMF)/sterile water (1:20) at 5 mg/mL for the crude extracts and 1 mg/mL for pure compounds for the screening. After incubation at 37°C for 24 hours, the inhibition zone corresponding to the halo (h) formed from well edge to the beginning of the zone of microbial growth was measured in millimeters (mm).

Table 1. List of target microorganisms used in antimicrobial activity test.

<table>
<thead>
<tr>
<th>Strain identification</th>
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<tbody>
<tr>
<td>Kocuria rhizophila ATCC 9341</td>
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<tr>
<td>Staphylococcus aureus ATCC 6538</td>
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<tr>
<td>S. aureus ATCC 25923</td>
</tr>
<tr>
<td>S. aureus penicillinase + (7+)</td>
</tr>
<tr>
<td>S. epidermidis 6epi</td>
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<tr>
<td>Streptococcus mutans ATCC 25175</td>
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<tr>
<td>S. mutans 11.1</td>
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<tr>
<td>S. sobrinus 180.3</td>
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<tr>
<td>Escherichia coli ATCC 10538</td>
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<tr>
<td>E. coli ec 26.1</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa ATCC 27853</td>
</tr>
<tr>
<td>P. aeruginosa 290D</td>
</tr>
<tr>
<td>Candida albicans ATCC 1023</td>
</tr>
<tr>
<td>C. albicans cas</td>
</tr>
<tr>
<td>C. tropicalis ct</td>
</tr>
</tbody>
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ATCC: American Type Culture Collection; a: Strain from collection of microbiology laboratory of FCFRP/USP

The minimal inhibitory concentration (MIC) of each isolated compound was determined using broth microdilution techniques to give a concentration between 1
and 0.01 mg/mL. In the tests, bacitracin (0.2 UI/mL) and ketoconazole (0.1 mg/mL) were used as positive controls for bacteria and fungi strains, respectively. Dimethylformamide (DMF)/sterile water (1:20, v/v) served as negative control for which no inhibitory effect could be observed. The tests were performed in duplicate for each microorganism evaluated with three replicates in each assay and the results were presented as the arithmetic average.

Reference
