Antimicrobial Screening and Quantitative Determination of Benzoic Acid Derivative of Gomphrena celosioides by TLC-Densitometry

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The genus Gomphrena (family Amaranthaceae) comprises approximately 120 species found in the Americas, Australia, and Indo-Malaysia; 46 species occur in Brazil, in savanna vegetation (cerrado), napeadic grassland (campo limpo), high altitude grassland (campo rupestre), and caatinga; only a few species are found in forest. A number of Brazilian Gomphrena species are employed in the treatment of bronchial affections, diarrhea, and fever, and as an analgesic, tonic, or carminative. This species show antimalarial and diuretic properties.1-3 There are few phytochemical and pharmacological screening reports on this genus. In this paper we deal with the isolation, structural elucidation of constituents, antimicrobial activity and quantitative determination of benzoic acid derivative by TLC-densitometry.

Experimental

Plant Material Gomphrena celosioides was collected in Paranaiba, Mato Grosso do Sul State, in December 1994 and authenticated by Prof. Josafá Carlos de Siqueira. A voucher specimen is deposited in the herbarium of Pontifical Catholic University, Rio de Janeiro (SCAB 4051).

Extraction and Purification Dry powdered aerial parts (6 kg) and roots (3 kg) were macerated successively with hexane, ethanol and methanol. The ethanolic extract of the aerial parts (281.0 g) was submitted to partition successively with hexane and chloroform yielding crude extract: 1.6 and 20.0 g respectively. The extracts were chromatographed respectively over a silica gel column chromatography using reducing pressure. The elution system followed the procedure above-mentioned. Fractions were collected and grouped according to the results of TLC. GC analysis of sterols were performed in a HP 5890 gas chromatograph equipped with a column HP-50 cross linked with 50% phenyl-methyl silicone (30 m×0.25 mm I.D.) and with an FID system. H2 was used as carrier gas at a flow-rate 39 cm/s. The injection split ratio was 1:60. The injection temperature was 260 °C. The column temperature was 280 °C and the detector temperature was 300 °C.

HPLC was performed in a LC-6A liquid chromatograph (Shimadzu) equipped with a UV-visible detector, using a Shin-Pack PREP-ODS column (20×250 mm, 5 μm, Shimadzu). 1H-NMR at 300 MHz and 13C-NMR at 75 MHz in deuterated solvent with TMS as internal standard.

Antimicrobial Testing Ten milligrams of each extract and pure compounds were suspended in 1 ml of DMSO. Twenty microliters of each suspension were used for testing.

The following strains were used as test organisms: Staphylococcus aureus (ATCC 12598), Salmonella typhi (ATCC 19430), Proteus mirabilis (ATCC 15290), Pseudomonas aeruginosa (ATCC 15442) and Escherichia coli (ATCC 8739).

Media, cultures of microorganisms and test plates were prepared according to Kirby-Bauer method. Inhibition zones were read after 18 to 24 h at 37 °C.

TLC-Densitometric Assay Different parts (stem, leaf, flower and root) of G. celosioides were collected in Ribeirão Preto (São Paulo State) and Paranaiba (Mato Grosso do Sul State), in March 2000. Air-dried material (200 mg d.w.) was extracted with methanol by 10 min (twice) in ultrasonic bath. They were prepared by dissolving of extract samples in 100 μl of methanol. Purified sample of 4-hydroxy-3-methoxy-benzoic acid (2), previously isolated of G. celosioides were used. Standard stock solution was prepared by dissolving 4.1 mg sample of 2 in 4.1 ml of methanol and diluted at 0.5, 0.1 and 0.05 mg/ml.

A Shimadzu high speed TLC-Scanner CS-930IPC was used with the following setting: beam size 0.4×0.4 mm, X= 25, Y=10, L= 3; AZS off, wavelength 247 nm. silica gel F254 (10×10 cm, 0.2 mm thick. Aldrich) plates were used. The mobile phase was n-ProH/EtOAc/H2O 12 : 9 : 6. Samples were applied with fixed volume pippetor (Gilson), at 10 mm from the lower edge of the plate. The mobile phase was allowed to run a distance of 100 mm in the saturated tank.

Diluted solutions were spotted on a TLC plate and developed under the above-mentioned conditions. The areas of the spots were then integrated by TLC-densitometry. Samples were spotted in triplicate. The calibration graph showed a linear relationship between the concentrations and the areas on
Results and Discussion

All compounds isolated from aerial parts and roots of *G. celosioides* have been found earlier in other *Gomphrena* species and our results corroborate the botanical classification of the plant from a chemotaxonomical point of view.

Fractionation of the aerial part extracts led to the isolation of 4-hydroxy-benzoic acid (1) and 4-hydroxy-3-methoxybenzoic acid or vanillic acid (2) besides of stigmaster, sitosterol and campesterol. Roots extracts yielded ecldosterone (3),4,5) methyl palmitate (4), stigmast-6-en-3-O-B-(D-glicopiranoside) (5),6) and stigmaster. To the best of our knowledge 1, 2, 4 and 5 were isolated for the first time from *G. celosioides.*

An *in vitro* antimicrobial screening of extracts and pure compounds of *G. celosioides* indicated positive activity against *Staphylococcus aureus* and *Salmonella typhi* but not effect was observed to *Pseudomonas aeruginosa*, *Proteus mirabilis* and *Escherichia coli* (Table 1).

The inhibitory action of phenolic compounds is due to the presence of phenolic–OH groups. It is known that the –OH group is much more reactive and can easily form hydrogen bonds with active sites of enzymes. Aziz *et al.* (1998) showed that vanillic acid (2) at the concentration of 0.4 mg/disc completely inhibited the growth of *E. coli.* However, in this work the concentration was used 0.2 mg/disc. Friedman *et al.* (2003) using different strains of *E. coli* and other bacteria evaluated the bactericidal activities of 34 benzoic acids and 35 benzaldehydes and compared their activity–structure relationships. Their results revealed the following order of bactericidal activity intensities by the phenolic compounds: trisubstituted OH > disubstituted OH > monosubstituted OH; and the concentration of benzoic acid derivatives that kills 50% of the bacteria was higher than 0.67%. According to Chamikha *et al.* (2002), high concentrations of p-hydroxybenzoic acid or vanillic acid inhibited growth, and decarboxylation could not occur completely, suggesting phenol or guaiacol toxicity, respectively. Neither growth nor decarboxylation occurred with concentrations higher than 130 mM (21.8 mg/ml) of vanillic acid or 20 mM (2.7 mg/ml) of p-hydroxybenzoic acid. Phenol and guaiacol were not metabolized and inhibited markedly growth of strain C2 (99% of similarity to *Escherichia coli*) at concentrations higher than 23 and 26 mM (2.1, 3.2 mg/ml), respectively. The above considerations were important to express ours results, in which the concentration of 10 mg/ml of ethanol extract and vanillic acid (2) were toxic for *S. aureus* and *S. typhi*. However, the same concentration was not toxic because it may have been metabolized by *P. aeruginosa*, *P. mirabilis* and *E. coli*.

Andersson *et al.* (1989) reported that steroidal glycosides are biologically active through antibacterial tests. This was the first time that stigmastane-type sterol was submitted to antibacterial activity.

For testing the quantitative accuracy of the TLC-densitometric method, the analysis of the reference substance and the extracts was repeated on triplicate. Separation of 2 was clear enough with a single development. Excellent linearity was observed between the concentration and area integrated by TLC-densitometry.

The relevant regression equation at 247 nm, was:

\[ y = 1941.2x - 36.979 \]

where *y* is the integration unit and *x* is the weight of 2 in milligrams per millilitre.

Results showed that young plants have high concentration with no significant difference in all parts analysed (Table 2). Major accumulation of 2 was observed in the flowers. Decreased production could be observed in roots plants grown in Paraíba, when we compare young and old plants grown in Ribeirão Preto. This compound accumulate in small quantities in stems of old plants.

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<tr>
<th>Microorganisms</th>
<th>Diameter of zone of inhibition (mm)</th>
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<tr>
<td></td>
<td>Ethanol extract</td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>7.6±2.5</td>
</tr>
<tr>
<td><em>P. aeruginosa</em></td>
<td>—</td>
</tr>
<tr>
<td><em>P. mirabilis</em></td>
<td>—</td>
</tr>
<tr>
<td><em>S. typhi</em></td>
<td>7.3±0.5</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>—</td>
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</tbody>
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Table 1. Antimicrobial Activity (Zone of Inhibition) Obtained with the Ethanol Extract of the Aerial Parts and Compounds Isolated of *G. celosioides*

<table>
<thead>
<tr>
<th>Materials</th>
<th>HMBA Contents (mg/g D.W.)</th>
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<tbody>
<tr>
<td>Flowers</td>
<td>YE-RP</td>
</tr>
<tr>
<td></td>
<td>OP-RP</td>
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<td>OP-PB</td>
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Table 2. Contents of 2 as Determined by TLC-Densitometry in Several Parts of *G. celosioides*

References
8) Gourma H. L. B., Tantawi L. B., El-Araki M., Benbye K. J., *ullerman*