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

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The antimicrobial activity of ethanolic extract and pure compounds of *Gomphrena celosioides* have been screened by Kirby–Bauer method. Quantitative determination of 4-hydroxy-3-methoxy-benzoic acid in stems, leaves, flowers and roots was established by TLC-densitometry. Results showed significant activity against *Staphylococcus aureus* and *Salmonella typhi*. There were no significant differences in the determined benzoic acid derivative.

Key words *Gomphrena celosioides*; Amaranthaceae; antimicrobial activity; 4-hydroxy-3-methoxy-benzoic acid; TLC-densitometry

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.[1)] A number of Brazilian *Gomphrena* species are employed in the treatment of bronchial affections, diarrhea, and fever, and as an analgesic, tonic, or carminative.[1)] This species show antimalarial and diuretic activities.[2,3)] There are few phytochemical and pharmacological screening report on this genus.[1,4)] 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 vacuum column filled with silica gel (silica gel H, Merck). The elution started with hexane by using vacuum. Then EtOAc was gradually added until the eluant was pure EtOAc. Then methanol was gradually added until the final eluant was pure methanol. Fractions were collected and grouped according to the results of TLC. The combined fractions of the hexanic extract were submitted to analysis by GC with authentic substances. Identification of stigmasterol, sitosterol and campesterol was carried out by comparing their retention times with those of the standards. Final separation of the chloroformic extract by HPLC on a silica gel RP-18 (20×250 mm) with MeOH : H_2O (3 : 7) gave 4-hydroxy-benzoic acid (**1**) and 4-hydroxy-3-methoxy-benzoic acid (**2**).

The methanolic extract of the roots (60.7 g) was submitted to partition successively with chloroform and *n*-butanol. The butanolic phase was evaporated and chromatographed over a column filled with silica gel (silica gel H, Merck). The elution started with hexane by using pressure. Then EtOAc was gradually added until the eluant was pure EtOAc. Then methanol was gradually added until the final eluant was pure methanol. One hundred and fifty milliliters fractions were collected and grouped according to the results of TLC. Final purification by PTLC on a silica gel C-18 (20×20 cm) eluting with MeOH : H_2O (3 : 7) gave ecdysterone (**3**).

The ethanolic extract of the roots (54.3 g) was fractionated by silica gel (silica gel G-60) column chromatography using reducing pressure. The elution system followed the procedure above-mentioned. Fractions were collected and grouped according to the results of TLC. Beginning fractions gave methyl palmitate (**4**) and others fractions were recrystallized with acetone to afford an saponin: stigmast-6-en-3-*O*-*β*-(D-glicopiranoside) (**5**). Another fraction was purified by silica gel (silica gel 60) column chromatography to afford stigmasterol.

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. H_2 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).

^{1}H-NMR at 300 MHz and ^{13}C-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 100μl 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-9301PC 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 60 F_{254} (10×10 cm, 0.2 mm thick, Aldrich) plates were used. The mobile phase was *n*-PrOH/EtOAc/H_2O 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

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each TLC plate. Aliquots (2 μl) of sample solutions were spotted on each TLC-plate, and after the development, the areas of the spot on the plate were integrated by TLC-densitometry.

Results obtained from TLC-densitometric analysis were expressed in terms of mean±S.E. values by paired *t*-test ($p<0.05$).

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-methoxy-benzoic acid or vanillic acid (**2**) besides of stigmasterol, sitosterol and campesterol. Roots extracts yielded ecdysterone (**3**),[4,5] methyl palmitate (**4**), stigmast-6-en-3-*O*-β-(D-glicopiranoside) (**5**)[5,6] and stigmasterol. 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.[7] It is known that the –OH group is much more reactive and can easily form hydrogen bonds with active sites of enzymes.[8] Aziz *et al.* (1998) showed that vanillic acid (**2**) at the concentration of 0.4 mg/disc completely inhibited the growth of *E. coli*.[7] However, in this work the concentration used was 0.2 mg/disc. Friedman *et al.* (2003) using differents strains of *E. coli* and others 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%.[9] According to Chamkha *et al.* (2002), high concentrations of *p*-hidroxybenzoic 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*-hidroxybenzoic 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.[10] 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 biologicaly active through antibacterial tests.[11] 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.

Table 1. Antimicrobial Activity (Zone of Inhibition) Obtained with the Ethanol Extract of the Aerial Parts and Compounds Isolated of *G. celosioides*

Microorganisms	Diameter of zone of inhibition (mm)[a]		
	Ethanol extract[b]	**2**[b]	**5**[b]
S. aureus	7.6±2.5	7.3±2.5	7.6±2.5
P. aeruginosa	—	—	—
P. mirabilis	—	—	—
S. typhi	7.3±0.5	7.3±0.5	7.3±0.5
E. coli	—	—	—

a) Values are mean±S.D. *b*) 10 mg/ml.

Table 2. Contents of **2** as Determined by TLC-Densitometry in Several Parts of *G. celosioides*

Materials	HMBA Contents (mg/g D.W.)			
	Flowers	Leaves	Stems	Roots
YP-RP	—	0.262±0.116	0.268±0.059	0.197±0.119
OP-RP	0.259±0.031	0.206±0.034	0.096±0.017	0.253±0.034
OP-PB	0.324±0.111	0.178±0.040	0.052±0.006	0.075±0.018

HMBA=4-hydroxy-3-methoxy-benzoic acid (**2**); YP-RP=young plants collected in Ribeirão Preto; OP-RP=old plants collected in Ribeirão Preto; OP-PB=old plants collected in Paranaiba; Values are mean±S.E., $n=3$.

Rf values was 0.9 approximately. The spot area of this compound was found to range linearly against measured concentrations. 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** were observed in the flowers. Decreased production could be observed in roots plants grown in Paranaiba, 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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