A New Heptasubstituted (*E*)-Aurone Glucoside and Other Aromatic Compounds of *Gomphrena agrestis* with Biological Activity

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A new aurone **1** and two known substances, aurantiamide acetate (**2**) and tiliroside (**3**), were isolated from the ethanolic extract of *Gomphrena agrestis*. The structural determination of **1** was based on spectroscopic and spectrometric data. The substance was defined as (*E*)-3′-*O*-*β*-D-glucopyranosyl-4,5,6,4′-tetrahydroxy-7,2′-dimethoxyaurone. Biological activity of the ethanolic crude extract and isolated compounds against bacteria, fungi and *Leishmania amazonensis* amastigotes was evaluated. This appears to be the first report documenting aurone and aurantiamide compounds in the Amaranthaceae family. In the evaluation of biological activity the ethanolic extract of *G. agrestis* and compounds **1**, **2**, and **3** were shown to be active mainly against *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Pseudomonas aeruginosa*.

Key words: Gomphrena agrestis, Aurone, Biological Activity

Introduction

The genus *Gomphrena*, which belongs to the tribe Gomphreneae of the Amaranthaceae family consists of approximately 120 species (Siqueira, 1994/1995). Many plants of this family are employed in folk medicine in the treatment of several diseases and for their nutritive value (Lewis and Elvin-Lewis, 1977; Siqueira, 1987; Macedo *et al.*, 1999; Pomilio *et al.*, 1994). *Gomphrena* spp. predominates in the Americas, particularly in South America where nearly 100 species are found in Brazil (Siqueira, 1984) and show species with antimicrobial activity used to treat gastrointestinal and respiratory disorders as well as infections diseases (Pomilio *et al.*, 1992; Siqueira, 1989; Buschi and Pomilio, 1983). Previous chemical studies with species of this genus were related to the isolation of hydrocarbons, alcohols, steroids, terpenoids, ecdysteroids, flavonoids and protoalkaloids (Savchenko *et al.*, 1998; Pomilio *et al.*, 1994; Buschi and Pomilio, 1983, 1982a, b; Buschi *et al.*, 1981, 1980, 1979). Despite some phytochemical and biological activity studies have been performed in certain species, overall the genus *Gomphrena* still remains poorly studied.

Gomphrena agrestis is a herbaceous plant commonly found in all Brazil and mainly in the "cerrado" and "campos rupestres" of Goiás, Minas Gerais, Mato Grosso and Bahia states (Siqueira, 1989). In our laboratory, several plant extracts from Amaranthaceae were screened *in vitro* against amastigote forms of *Leishmania amazonensis* and for antimicrobial activity. In a preliminary evaluation, the ethanolic crude extract (dry, whole plant) of *G. agrestis* has shown good antimicrobial and leishmanicidal activities. Therefore, in the course of chemical and biological investigations of Amaranthaceae plants, this work reports the isolation and identification of a new aurone **1** and two known substances, aurantiamide acetate (**2**) and tiliroside (**3**), isolated from the ethanolic extract of *G. agrestis*. The ethanolic crude extract and compounds **1**, **2** and **3** were screened for antimicrobial activity *in vitro* as well as their toxicity against *L. amazonensis* amastigotes from axenic

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culture were evaluated. This is the first report documenting **1** and **2** and their biological activity in the Amaranthaceae family.

Material and Methods

General experimental procedures

The ^1H and ^{13}C NMR and 2D NMR were recorded in MeOH-d_4 on a Bruker DPX-300 (300 MHz), DPX-400 (400 MHz) or DPX 500 (500 MHz). ESI-MS was measured on a Micromass Plataform VG–II. HPLC analysis was carried out on an ODS-Shimpack RP-18 column [4.6 × 250 mm, 5 μm; MeOH/H_2O 40:60 v/v (for **1**) and MeOH/H_2O 50:50 v/v (for **3**); λ = 280 nm; flow 1 ml/min; UV detector SPD-6AV] and ODS-Shimpack RP-18 column (20 × 250 mm, 5 μm; MeOH/H_2O 40:60 v/v; λ = 280 nm; flow 8 ml/min). IR spectra were recorded on a Perkin-Elmer 1420. UV spectra were recorded on an U-3501 Spectrophotometer. In the biological assay, MTT cleavage was measured by using a multiwell scanning spectrophotometer (model #3550; Bio-rad Laboratories, Richmond, CA) with a reference wavelength of 655 nm and a test wavelength of 595 nm.

Plant material

Gomphrena agrestis Moq. was collected at Alto Paraiso, Goiós, Brazil, in July 1995, and identified by Professor Josafa Carlos de Siqueira (Pontifícia Universidade Católica, Rio de Janeiro). A voucher specimen was deposited at the Nova Friburgo Herbarium under number SCAB-4054.

Extraction and isolation of compounds

The whole plants of *G. agrestis* were dried in an open stove at 40 °C and powdered affording 1300 g. Exhaustive extraction with ethanol after hexane extraction gave 37 g of the crude extract (dry weight).

To obtain the aurone **1**, 36.0 g of the crude ethanolic extract of *G. agrestis* (whole plant) was partitioned. After subsequent extractions with hexane, followed by CH_2Cl_2, the hydroalcoholic phase was column filtered with amberlite XAD-2. The ethanolic phase (4.5 g) obtained from the filtration was column fractionated with Sephadex LH-20 eluted with MeOH and yielded 14 samples. After being subjected to a HPLC separation the 11th sample yielded 20 mg of compound **1** and the 14th sample yielded 28 mg of compound **3**. Aurantiamide acetate (**2**) was obtained from the dichloromethane phase (4.6 g). Fractionation on VLC (Kieselgel 60H, 500.0 g) eluted with a gradient of hexane, EtOAc and MeOH yielded 9 samples. The 6th sample was column fractionate with Sephadex LH-20 eluted with MeOH/$CHCl_3$ (50:50 v/v) and yielded 5 samples of which the 2nd was subjected to PTLC (MeOH/$CHCl_3$ 50:50), yielding 8 mg of compound **2**.

(*E*)-3′-O-β-D-glucopyranosyl-4,5,6,4′-tetrahydroxy-7,2′-dimethoxyaurone (**1**): Yellow gum. – M.p. 138–140 °C. – IR (MeOH film): ν_{max} = 3360, 2940, 1645, 1550, 1510, 1460, 1420, 1360, 1280, 1190, 1070 cm^{-1}. – UV (MeOH): λ_{max} = 254, 268 sh, 348 nm; (NaOMe): λ_{max} = 269, 334, 411 nm; (NaOAc): λ_{max} = 271, 316, 376 nm; (H_3BO_3): λ_{max} = 270, 353 nm.

Biological activities

Antimicrobial activity: 22 strains, including gram positive and gram negative bacteria and yeast strains, were used for the susceptibility assays. The following microorganisms were used: *Escherichia coli* – ATTC 10538; *E. coli* – ec 26.1 (field strain); *Pseudomonas aeruginosa* – ATCC 27853; *P. aeruginosa* – 290D (field strain); *Micrococcus luteus* – ATTC 9341; *Staphylococcus aureus* – ATTC 25923, 6538 and 25213; *S. aureus* 7+ penicilinase producer; *S. aureus* 8– penicilinase non-producer; *Staphylococcus epidermidis* 6epi, epiC (field strains), cultivated for 24 h at 37 °C in Mueller Hinton broth (MHb) (Difco); *Enterococcus faecalis* – ATCC 10541; *Streptococcus mutans* – ATCC 25175; *S. mutans* (strains Fab 3; 9.1; 11.1; 11.22.1) and *Streptococcus sobrinus* 180.3 (field strains), incubated for 24 h at 37 °C in Brain Heart Infusion (BHI) (Difco); *Candida albicans* – ATTC 1023; *C. albicans* cas (field strain) and *Candida tropicalis* ct (field strain), incubated for 24 h at 37 °C in Sabouraud glucose agar slants. The standard and field strains (isolated of patients) were collected from "Departamento de Análises Clínicas, Toxicológicas e Bromatológicas, Faculdade de Ciências Farmacêuticas de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto, SP, Brasil".

Sensitivity tests were performed using a modified agar-well diffusion method (well technique in double layer) according to Salvador *et al.* (2002), Okeke *et al.* (2001), Cole (1994) and Grove and Randall (1955). The test bacterial and fungi strains were inoculated into Mueller Hinton Medium

(MH) (Difco) agar plates (*Escherichia*, *Pseudomonas*, *Micrococcus*, *Staphylococcus* and *Candida* strains) and Brain Heart Infusion Agar (BHIa) (Difco) plates (*Enterococcus* and *Streptococcus* strains), containing an inoculum size of 10^6 cfu/ml (0.5 McFarland scale). The inoculum size of each test strain was standardized according to the National Committee for Clinical Laboratory Standards (NCCLS, 1993).

A volume the 20 μl of each test-drug solution prepared in propylene glycol/RPMI-1640 (1:9) at 1 mg/ml for the ethanolic crude extract and isolated compounds was applied to 5.0 mm diameter wells in the screening. After incubation at 37 °C for 24 h, the inhibition zone corresponding to the halo (H) formed from the well edge to the beginning of the region of microbial growth was measured in millimeters (mm).

The minimum inhibitory concentration (MIC) was determined for isolated compounds (Okeke *et al.*, 2001; Okunji *et al.*, 1990) evaluated in the range 0.02 to 0.5 mg/ml. In these tests, bacitracine (0.2 UI/ml) and ketoconazole (0.1 mg/ml) were used as experimental positive controls for strains analyzed, while propylene glycol/RPMI-1640 (1:9) served as the negative control. The bioassays were performed in duplicate for each microorganism evaluated.

Leishmanicidal activity: The effect of crude extract and isolated compounds on viability of *L. amazonensis* (strain designation MPRO/BR/72/M 1841) amastigotes was performed using parasites cultured according to established protocols (Pral *et al.*, 2003; Salvador *et al.*, 2002) and incubated at 33 °C for 18 h with the ethanolic crude extract (1 mg/ml) or isolated compounds (0.02, 0.1 and 0.5 mg/ml) dissolved previously in dimethyl sulfoxide (DMSO)/RPMI-1640 (1:99). 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. Negative and positive controls containing, respectively, DMSO/RPMI-1640 (1:99) and amphotericin B (0.02 mg/ml) were run in parallel. The bioassays were performed in triplicate.

Table I. ^{1}H (400 MHz) and ^{13}C NMR (100 MHz) data for compound **1** (MeOH-d_4; δ in ppm; J in Hz), including 2D HMQC and HMBC heteronuclear correlations.

Position	δ_H	m, J	δ_C	HMBC ($^3J_{CH}$ coupling)
2	–	–	148.8	–
3	–	–	179.9	–
4	–	–	–	–
5	–	–	133.2	–
6	–	–	154.2	–
7	–	–	148.8	–
8	–	–	159.1	–
9	–	–	106.4	–
10	7.80 (1H)	s	114.7	H10/C-2*; /C-2′; /C-6′
1′	–	–	123.4	–
2′	–	–	133.2	–
3′	–	–	135.4	–
4′	–	–	151.2	–
5′	6.79 (1H)	d, 8.2	116.4	H5′/C-1′; /C-3′
6′	7.46 (1H)	d, 8.2	124.3	H6′/C-2; /C-10
1″	5.28 (1H)	d, 6.8	104.1	H1″/C-3′
2″	3.51 (2H)	m	76.3	–
3″	3.28 (1H)	m	78.9	–
4″	3.37 (1H)	m	71.9	–
5″	3.51 (2H)	m	78.4	–
6″a	3.74 (1H)	dd, 1.8; 12.2	62.9	–
6″b	3.58 (1H)	dd, 5.2; 12.2	–	–
7-OMe	3.83 (3H)	s	57.2	7-OMe/C-7
2′-OMe	3.76 (3H)	s	61.4	2′-OMe/C-2′
4-OH	6.36 (1H)	s		4-OH/C-3**; /C-5; /C-6; /C-8; /C-9

– Non-assigned signal.
* $^2J_{CH}$ coupling.
** $^4J_{CH}$ coupling.

Results and Discussion

The ^{1}H NMR spectrum of the aurone showed signals indicative of the presence of *ortho*-coupled aromatic protons (δ 7.46, 8.2 Hz, 1H and δ 6.78, 8.2 Hz, 1H), methoxyl protons (δ 3.83, s, 3H and δ 3.76, s, 3H) and a β-anomer proton (δ 5.27, d, 6.8 Hz, 1H). The presence of a total of 20 signals in the ^{13}C NMR spectrum and analysis of the IR region with the presence of bands of absorption at 3360 cm^{-1} (*br*, hydroxyl group), 1645 cm^{-1} (cyclic ketone) and 1600 to 1500 cm^{-1} (aromatic ring) indicate that the substance could belong to the flavonoid class. Correlation of the singlet at δ 7.80 (1H) with the signal at δ 114.7 in the HMQC experiment indicates the association with an aurone. The *E*-olefinic configuration was defined by the chemical shift value of carbon atom C-10 that is found more deshielded in the *E*-aurones (Agrawal, 1989). The ^{1}H and ^{13}C NMR data, NOE experiment, UV shifts, and the HMQC and HMBC correlations contributed in establishing the substitution pattern and the position of methoxyl groups and glycosyl moiety in the aurone. The ^{1}H NMR spectrum analysis indicated a completely substituted A ring and a trisubstituted B ring. The irradiation of the signal at δ 3.83 caused a large increase in signal intensity at δ 7.80 (H-10), suggesting that the methoxyl group can be located at position C-2′ of the B ring. This can be confirmed by the shift value of C-10, which occurs at δ 114.7. The value of this carbon atom in the *E*-aurones falls between δ 119.8 and 122.2 and the presence of a 2′-oxysubstituent brings this carbon atom close to δ 5 upfield shifted (Agrawal, 1989). Furthermore, in the HMBC experiment it was possible to observe the $^3J_{CH}$ coupling correlations between the signal of the protons of the methoxyl group and the C-2′ (δ 3.83–δ 133.2), as well as between the signal of the exocyclic olefinic methine H-10 and the C-2′ (δ 7.80–δ 133.2). UV spectrum analysis in the presence of NaOMe showed a bathochromic shift of 60 nm in band I. This dislocation indicated the presence of a free hydroxyl group at positions C-6 and C-4′, which was confirmed by the UV test with NaOAc (bathochromic shift of 28 nm in band I).

Thus, there must be a 2′,3′,4′-trisubstituted B ring, with a methoxyl group at C-2′ position and a hydroxyl group at C-4′ position. A bathochromic shift of 5 nm in band I in the UV experiment with MeOH in the presence of NaOAc and H_3BO_3 showed the absence of a free *ortho*-dihydroxyl group in the B ring. Such considerations suggested that sugar is associated at position C-3′. In the HMBC experiment it was possible to observe δ 5.27–δ 135.4 (H-1″-C-3′) and δ 6.79–δ 151.2 (H-5′-C-4′) $^3J_{CH}$ coupling correlations. The type of sugar was determined by comparing the observed resonances (^{1}H and ^{13}C NMR data) with those of model glycosylated flavonols and a β configuration was evidenced by the coupling constant (J = 6.8 Hz: 1″–2″ diaxial coupling) of the anomeric hydrogen (Agrawal, 1989). The location of the methoxyl group at δ 3.76 was suggested on the basis of UV spectrum analysis, which exhibited a value of λ_{MeOH} of 348 nm. The long wavelength absorption in aurones falls in the area of 400 nm. However, a hypsochromic shift is observed if a 7-hydroxyl group in the 6,7-dihydroxy-aurone is methoxylated (Mabry *et al.*, 1970). Analysis of the UV experiment with the inclusion of NaOMe and NaOAc, respectively, showed a bathochromic shift of 60 and 28 nm in band I, indicating the presence of a free hydroxyl group at position C-6. The ba-

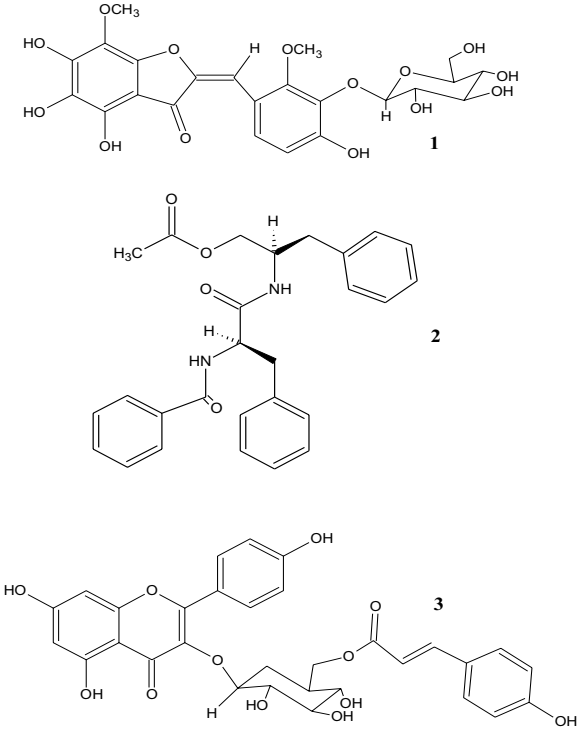

Fig. 1. Structures of compounds (*E*)-3′-*O*-β-D-glucopyranosyl-4,5,6,4′-tetrahydroxy-7,2′-dimethoxyaurone (**1**), aurantiamide acetate (**2**) and tiliroside (**3**) isolated from *Gomphrena agrestis*.

thochromic shift of 5 nm in band I in the UV experiment in the presence of NaOAc and H_3BO_3 indicates the existence of the free *ortho*-dihydroxyl group in the A ring. Furthermore, the signal shown at δ 6.36 (s, 1H) in the ^{1}H NMR spectrum, which disappears with the addition of D_2O, was attributed to the signal of the hydrogen of the hydroxyl group located at position 4′. In contrast to what is seen with other flavonoids, the 4-hydroxyl function, which is biosynthetically equivalent to position C-5 in normal flavonoids (Harborne, 1994), is weakly hydrogen-bonded to the carbonyl group (Mabry *et al.*, 1970). Thus, taking into account such considerations, the A ring is 4,5,6,-trihydroxy-7-methoxy-substituted. The correlation of the 4-OH signal (δ 6.36) with quaternary carbon atoms in the HMBC experiment (Table I) assisted in assigning the carbon atoms of the A ring. The combined analysis of all experiments, the comparison of the observed resonances with those of model flavonoids (Saturnino *et al.*, 1997; Harborne, 1994; Agrawal, 1989), and taking into consideration the known effect of the substitution on the flavonol ring (Agrawal, 1989), the structure of the aurone was determined as (*E*)-3′-*O*-β-D-glucopyranosyl-4,5,6,4′-tetrahydroxy-7,2′-dimethoxyaurone (Fig. 1). The peak reading at *m/z* 547 $[M + Na]^+$ in ESI-MS is in agreement with the expected formula $C_{23}H_{24}O_{14}$ (M_R 524) of the substance.

The identification of aurantiamide acetate (**2**) and tiliroside (**3**) (Fig. 1) has been published previously (Kaouadji, 1990; Banerji and Ray, 1981), and authors may provide copies of original spectra.

Initial observations indicated the activity of the crude ethanolic extract of *G. agrestis* (whole plant) against bacteria and *L. amazonensis*. This extract inhibited the growth of the gram positive bacteria as *S. aureus* ATTC 25923 and *S. epidermidis* (strains 6epi and epiC) with inhibition zones of 8, 6 and 6 mm, respectively, and of the gram negative *P. aeruginosa* strains ATTC 27853 and 290D with inhibition zones of 7 and 6 mm, respectively. The compounds **1**, **2** and **3** were obtained from this extract, which results are showed in Table II. These isolated compounds were active mainly against strains of *Staphylococcus aureus*, *S. epidermidis* and *Pseudomonas aeruginosa* with MIC values between 0.1 and 0.5 mg/ml. While the crude ethanolic extract markedly reduced the viability of *L. amazonensis* amastigotes to 1.7% (at 1 mg/ml), the isolated compounds did not appreciably interfere with parasite viability at 0.5 mg/ml. This latter result is in agreement with previous findings with this class of compounds (aurones), indicating that the introduction of bulky substituents (benzylation or glycosylation), oxygenation pattern of the aromatic ring and structural rigidity correlate with reduction of leishmanicidal activity (Kayser *et al.*, 2002, 1999). Therefore, subsequent fractionation and purification resulted in substances displaying low activity against *L. amazonensis* amastigotes compared to the ethanolic extract. This is fairly frequent when a chemical study is accompanied by evaluation of biological activity, and can be related to various factors, including loss of activity during the isolation procedure and/or synergism (Hostettmann *et al.*, 1998).

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Table II. Antibacterial and antifungal activities of the ethanolic crude extract and isolated compounds from *Gomphrena agrestis*.

Microorganism	Tested material								
	Ethanolic extract	**1**		**2**		**3**		P[c]	N[d]
	H	H	MIC	H	MIC	H	MIC	H	H
Micrococcus luteus (ATTC 9341)[a]	–	–		–		–		25	–
Staphylococcus aureus (ATCC 6538)[a]	–	–		–		–		23	–
Staphylococcus aureus (ATTC 25213)[a]	–	–		–		–		29	–
Staphylococcus aureus (ATTC 25923)[a]	8	6	> 0.5 (1.0)	8	0.1 (0.2)	6	0.5 (0.8)	29	–
Staphylococcus aureus penicilinase + (7+)[b]	–	–		–		6	0.5 (0.8)	25	–
Staphylococcus aureus penicilinase – (8–)[b]	–	–		–		–		25	–
Staphylococcus epidermidis (6epi)[b]	6	7	0.1 (0.2)	6	0.5 (1.1)	6	> 0.5 (0.8)	31	–
Staphylococcus epidermidis (epiC)[b]	6	6	0.5 (1.0)	6	0.5 (1.1)	–		30	–
Streptococcus mutans (ATTC 25175)[a]	–	–		–		–		24	–
Streptococcus mutans (Fab 3)[a]	–	–		–		–		22	–
Streptococcus mutans (11.1)[b]	–	–		–		–		22	–
Streptococcus mutans (9.1)[b]	–	–		–		–		22	–
Streptococcus mutans (11.22.1)[b]	–	–		–		–		24	–
Streptococcus sobrinus (180.3)[b]	–	–		–		–		23	–
Enterococcus faecalis (ATTC 10541)[a]	–	–		–		–		28	–
Escherichia coli (ATTC 10538)[a]	–	–		–		–		32	–
Escherichia coli (ec 26.1)[b]	–	–		–		–		32	–
Pseudomonas aeruginosa (ATTC 27853)[a]	7	6	0.5 (1.0)	7	0.5 (1.1)	6	> 0.5 (0.8)	22	–
Pseudomonas aeruginosa (290D)[b]	6	6	0.5 (1.0)	6	0.5 (1.1)	–		22	–
Candida albicans (ATTC 1023)[a]	–	–		–		–		30	–
Candida albicans (cas)[b]	–	–		–		–		30	–
Candida tropicalis (ct)[b]	–	–		–		–		15	–

H: Halo (diameter) of inhibition.
–: Without inhibition of the development at 1 mg/ml.
MIC: Minimum inhibitory concentration in mg/ml (mM).
[a] Standard strain.
[b] Strain of collection of microbiology and mycology laboratories of the "Faculdade de Ciências Farmacêuticas de Ribeirão Preto, Universidade de São Paulo (FCFRP/USP), Ribeirão Preto-SP, Brasil".
[c] Positive experimental control, bacitracine (0.2 UI/ml) for bacteria strains and ketoconazole (0.1 mg/ml) for yeasts strains.
[d] Negative experimental control, propylene glycol/RPMI-1640 (1:9).
All experiments were run in duplicate.

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