Bioactive Phenolic Compounds from Aerial Parts of *Plinia glomerata*

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The present work describes the antinociceptive properties and chemical composition of the aerial parts of *Plinia glomerata* (Myrtaceae). Both of the extracts evaluated, acetonic and methanolic, showed potent antinociceptive action, when analyzed against acetic acid-induced abdominal constrictions in mice, with calculated ID_{50} (mg/kg, i. p.) values of 24.8 and 3.3, respectively. Through usual chromatographic techniques with an acetonic extract, the following compounds were obtained: 3,4,3′-trimethoxy flavellagic acid (**1**), 3,4,3′-trimethoxy flavellagic acid 4′-*O*-glucoside (**3**) and quercitrin (**4**), which were identified based on spectroscopic data. Compounds **1** (ID_{50} = 3.9 mg/kg, i. p., or 10.8 μmol/kg) and **3** (ID_{50} = 1.3 mg/kg or 2.5 μmol/kg) were notably more active than some well-known analgesic drugs used here for comparison.

Key words: Plinia glomerata, Antinociception, Phenolic Compounds

Introduction

Several plants belonging to the Myrtaceae family are used in traditional medicine as medicinal agents, *e.g.* as antirheumatics, antidiabetics, antimicrobials, diuretics, and digestive system regulators, and the literature reports that several of these plants exhibit promising biological effects in different experimental models (Gibbons, 2003; Bnouham *et al*., 2006). *Plinia glomerata* (Myrtaceae) is a Brazilian native plant, which is widely distributed in the south of Brazil and is known as "cabeludinha" or "yellow jaboticaba". However, few chemical and pharmacological studies have been reported for the genus *Plinia*.

Previous preliminary studies carried out by our group with *P. glomerata* demonstrated that this plant has compounds with interesting biological effects (Serafin *et al.,* 2005). This led us to study this plant in more detail. Thus, the present paper describes the isolation of phenolic compounds from *P. glomerata* aerial parts and the evaluation of the possible analgesic effect of the extracts, fractions and some pure compounds using the writhing test in mice.

Material and Methods

Plant material

Aerial parts of *P. glomerata* were collected in Epagri/Itajaí (State of Santa Catarina, Brazil) during spring 2005 (September) and a voucher specimen (VC Filho 052) was deposited at the Barbosa Rodrigues Herbarium (BRH), Itajaí, Santa Catarina, Brazil. The plant was authenticated by Prof. Oscar Benigno Iza (BRH and UNIVALI).

Preparation of extracts and isolation of constituents

Dried aerial parts of *P. glomerata* (1.7 kg) were cut into small pieces and macerated in acetone for 10 d and then in methanol for 3 d. The solvent was concentrated under reduced pressure to give the respective extracts of acetone (110 g) and methanol (60 g). Part of the acetonic extract (30 g) was chromatographed using a silica gel column eluted with a mixture of $CHCl_3$/MeOH with increasing polarity, yielding 255 fractions of 10 ml each. The fractions were monitored by TLC and combined according to their similarities. Fractions 49–150 were combined, yielding 150 mg of a pure yellow

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powder (**1**). Fractions 160–255 were also combined, yielding 139 mg of a pure white powder (**3**).

The spectral data (IR, ^{1}H NMR, ^{13}C NMR, Hetcor, NOE, INEPT) allowed us to conclude that the compounds are 3,4,3′-trimethoxy flavellagic acid (**1**) and 3,4,3′-trimethoxy flavellagic acid 4′-*O*-glucoside (**3**), respectively. Another part of the acetonic extract (10 g) was dissolved in dichloromethane, furnishing the DCM-soluble fraction (1.3 g) and an unsoluble fraction. Part of the unsoluble fraction (5 g) was chromatographed on a silica gel column using a $CHCl_3$/MeOH gradient as eluent, yielding 69 fractions of 10 ml each. Fractions 45–52 were combined (614 mg) because they were very similar on TLC, showing two spots. They were chromatographed using Sephadex LH-20 as stationary phase and a mixture of MeOH/H_2O (8:2) as eluent, yielding 20 fractions of 3 ml each. Fractions 3–5 yielded 11 mg of compound **3**, while fractions 7–11 furnished 33 mg of a yellow substance, identified as quercetin-3-*O*-rhamnoside (quercitrin) (**4**), which was confirmed by direct comparison with an authentic sample.

General experimental procedures

The melting points were determined with a MQ APF-302 (Microquímica) apparatus and are uncorrected. ^{1}H (400 MHz) and ^{13}C (75 MHz) NMR spectra were obtained using a Varian Xl 400 spectrometer. Mass spectra were obtained by electrospray ionization (ESI) on a Thermo Finnigan LCQ DECA XP Plus ion-trap mass spectrometer. IR spectra (KBr) were recorded on a Bomem 100 FT/IR and UV spectra on a Cary spectrometer.

3,4,3′-Trimethoxy flavellagic acid (**1**): Yellow solid; m.p. 278–280 °C [lit. m.p. 278–280 °C (Row and Raju, 1967)]. – UV: λ = 247, 364 (sh), 383 nm; (+ NaOAc): λ unaltered; (+ $AlCl_3$): λ = 245, 411 nm. – IR (KBr): ν = 3606, 1712, 1611, 1510, 1080 cm^{-1}. – ^{1}H NMR ($CDCl_3$): δ = 7.75 (H-5′, s), 4.20, 4.18, 3.97 (OMe × 3 ss). – ^{13}C NMR: see Table I. – ESI$^-$ MS: *m/z* = 359 [M–H]$^-$.

3,4,3′-Trimethoxy flavellagic acid 4′-O-glucoside (**3**): White solid; m.p. 257–259 °C [lit. m.p. 258–261 °C (Adigun *et al.*, 2000)]. – UV: λ = 240, 318 (sh), 372 nm; (+ MeOH): unaltered; (+ $AlCl_3$): λ = 245, 410 nm. – IR (KBr): ν = 3340, 1724, 1695, 1616, 1482, 1450, 1308, 1082 cm^{-1}. – ^{1}H NMR (DMSO-d_6/$CDCl_3$ 1:1; 50 °C): δ = 7.80 (H-5′, s), 5.08 (H-1′′, d, J = 7.5 Hz), 4.16, 4.12, 3.92 (OMe × 3, ss), 3.72 (H-6′′, br d), 3.56 (H-6′′, dd), 3.43–3.30 (H-2′′–H-5′′, m). – ^{13}C NMR: see Table I. – ESI$^-$ MS: *m/z* = 521 [M–H]$^-$; ESI$^+$ MS (rel. int.): *m/z* = 1067 (100) [2M+Na]$^+$, 545 (10) [M+Na]$^+$.

Acid hydrolysis: **3** (30 mg) was refluxed in aqueous H_2SO_4 (7%, 5 ml) for 2 h, the solution cooled and extracted with EtOAc. The resulting residue (20 mg) from the organic layer was identical to **1**.

Pharmacological assays

Male Swiss mice, 25–35 g, were kept in a temperature-controlled environment [(23 ± 2) °C] with a 12 h light dark cycle. Food and water were freely available.

Abdominal constriction was induced by intraperitoneal injection of acetic acid (0.6%) according to the procedure described previously (Collier *et al.*, 1968). The animals were pretreated intraperitoneally (30 min before) with the acetonic (10, 20 and 30 mg/kg) or methanolic (3, 6 and 10 mg/kg) extracts and compounds **1** and **3** (1, 3 and 10 mg/kg) obtained from *P. glomerata* before injection of acetic acid. The control animals received the same volume of 0.9% NaCl solution (10 ml/kg) and all experiments were performed at 20–22 °C. After the challenge, pairs of mice were placed in separate boxes and the number of abdominal constrictions was cumulatively counted over a period of 20 min. Antinociceptive activity was expressed as the reduction of the number of abdominal constrictions of mice pretreated with extracts and pure compounds compared to control animals.

Statistical analysis

The results are presented as mean ± s. e.m., and statistical significance between groups was determined by analysis of variance using Dunnett's multiple comparison test. P values less than 0.05 ($P < 0.05$) were considered as indicative of significance. The mean ID_{50} values (the dose of extracts or compounds, which reduced responses by 50% relative to control values) were estimated by linear regression from individual experiments using "GraphPad Software".

Results and Discussion

Compound **1** was isolated as a yellow solid, m.p. 278–280 °C and the molecular formula $C_{17}H_{12}O_9$ was assigned on the basis of a peak at 359 [M–H]$^-$ in the ESI negative mass spectrum, which was corroborated by the presence of seventeen signals in the ^{13}C NMR spectrum. These signals included

Table I. ^{13}C NMR data for compounds **1** (in $CDCl_3$), **2** (in DMSO-d_6) and **3** (in $CDCl_3$/DMSO-d_6).

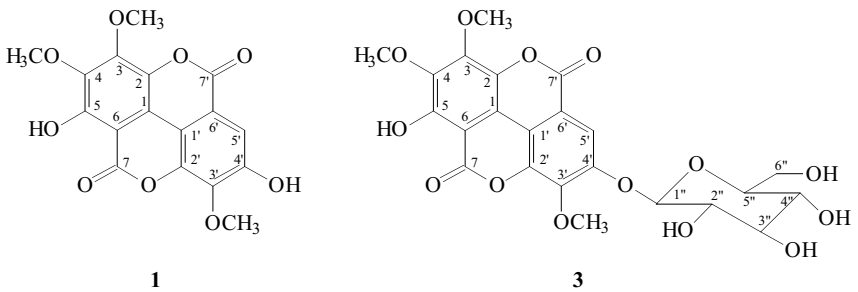
C	2	1	3
1	111.2[a]	111.9[a]	112.8[a]
2	141.2[b]	140.8[b]	141.5[b]
3	140.5[b]	152.4	153.0
4	153.2[c]	134.1	134.5
5	107.7	147.7	147.8
6	111.2[a]	98.1	98.1
7	158.7[d]	161.6	161.9
1′	112.7[a]	113.6[a]	113.7[a]
2′	141.7[b]	141.3[b]	141.9[b]
3′	141.0[c]	142.0	142.5
4′	153.9[c]	153.8	153.0
5′	111.9	112.4	113.0
6′	113.6[a]	114.0	114.3
7′	158.5[d]	159.0	158.7
OMe	61.5	62.4	62.3
OMe	61.1	61.8	62.2
OMe	56.9	61.7	61.7
1′′			102.1
2′′			77.9
3′′			77.2
4′′			73.9
5′′			70.2
6′′			61.3

[a–d] Values in the same column may be interchanged.

those for two carbonyl ester or lactone groups (δ_C 161.6 and 159.0 ppm), three *ortho-ortho* disubstituted methoxy groups (δ_C 62.4, 61.8 and 61.7 ppm), one methine group (δ_C 112.4 ppm) and eleven quaternary carbon atoms (Table I). Accordingly, the IR spectrum exhibited absorption(s) at 1712 cm^{-1} for carbonyl groups, and the ^1H NMR exhibited only four singlets at δ_H 3.97, 4.18, 4.20 and 7.75 ppm for the three methoxy groups and one aromatic proton, respectively. Since the three OMe groups may be only substituents, a flavellagic acid carbon skeleton was assumed for compound **1**. Comparison of its NMR data (Table I) with those of 3,4,3′-trimethoxy ellagic acid (**2**), a compound previously isolated in our laboratory (unpublished results), indicated that the only difference was the presence of a second methine group in the latter. The location of the *ortho-ortho* disubstituted methoxy groups was confirmed by the UV spectrum, whose maximal absorptions were shifted by adding $AlCl_3$, but not by NaOAc. Therefore, compound **1** is 3,4,3′-trimethoxy flavellagic acid (Fig. 1), firstly isolated from *Terminalia paniculata* (Row and Raju, 1967) and more recently from *Ruprechtia tangarana* (Pettit *et al.*, 2003).

Compound **3** (ESI$^-$ MS: m/z = 521 $[M-H]^-$) was a glucoside of **1**, as supported by the appropriate signals for the sugar in the ^{13}C NMR spectrum, and its location on position 4′ was established early by the difference NOE experiment, because the irradiation of the aromatic proton (H-5′) at δ 7.80 ppm enhanced the signal of the anomeric proton (δ 5.08 ppm). Finally, selective INEPT experiments enabled us to assign most of the carbon signals in Table I. The irradiation of H-5′ gave a response to signals at δ 158.7, 153.0, 142.5 and 114.3 ppm which may be attributed to C-7′ (^3J), C-4′ (^2J), C-3′ (^3J) and C-6′ (^2J), respectively; conversely, the irradiation of the anomeric proton (H-1′′) enhanced only the signal at δ 153.0 ppm (C-4′, ^3J). Therefore, compound **3** is 3,4,3′-trimethoxy flavellagic acid 4′-*O*-glucoside (Fig. 1), also isolated from *Anageissus leocarpus* (Adigun *et al.*, 2000). Hydrolysis of this compound confirmed the structure of compound **1**.

The pharmacological evaluation of both acetonic and methanolic extracts from *P. glomerata* aerial parts showed that they exhibit pronounced and dose-dependent inhibition against the writhing test when administered by the intraperitoneal



1 **3**

Fig. 1. Molecular structures of 3,4,3′-trimethoxy flavellagic acid (**1**) and 3,4,3′-trimethoxy flavellagic acid 4′-*O*-glucoside (**3**).

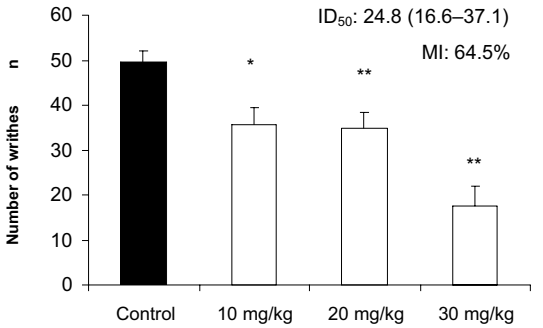


Fig. 2. Antinociceptive effects of an acetonic extract from *P. glomerata* injected intraperitoneally against writhing in mice. Each group represents the mean of 8 to 10 experiments and the vertical bars indicate the s.e.m. * $P < 0.05$; ** $P < 0.01$.

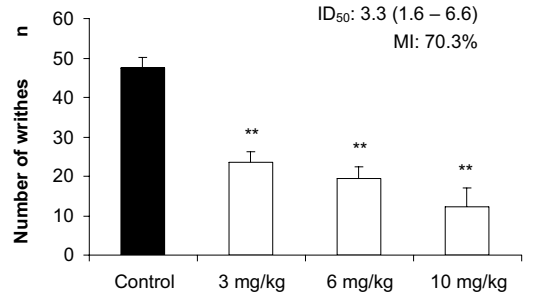


Fig. 3. Antinociceptive effects of a methanolic extract from *P. glomerata* injected intraperitoneally against writhing in mice. Each group represents the mean of 8 to 10 experiments and the vertical bars indicate the s.e.m. * $P < 0.05$; ** $P < 0.01$.

route. The calculated ID_{50} (mg/kg) values were 24.8 and 3.3, with maximum inhibition of 64.5 and 70.3% for acetonic and methanolic extracts, respectively (Figs. 2 and 3).

Table II. Antinociceptive activity of some reference drugs and 3,4,3′-trimethoxy flavellagic acid (**1**) and 3,4,3′-trimethoxy flavellagic acid 4′-*O*-glucoside (**3**) isolated from *P. glomerata* against acetic acid-induced abdominal constriction in mice.

Compound[a]	ID_{50} [μmol/kg, i. p.][b]	MI (%)[c]
1	10.8 (5.5– 21.3)	97 ± 0.5
3	2.5 (1.8– 3.5)	94 ± 1.0
Aspirin	133.1 (73.0–243.3)	83 ± 1.4
Paracetamol	125.8 (105.9–152.3)	88 ± 1.0
Dipyrone	162.0 (88.0–296.0)	54 ± 2.0
Diclofenac	38.0 (29.5– 49.0)	93 ± 3.0

Each group represents the mean ± s.e.m. of 6 to 10 experiments.
[a] Aspirin, acetyl salicylic acid (Bayer); paracetamol, *N*-acetyl-*p*-aminophenol (Jansen-Cilag); dipyrone, [(2,3-dihydro-1,5-dimethyl-3-oxo-2-phenyl-1*H*-pyrazol-4-yl)-methylamino] methane sulfonic acid sodium salt mono hydrate (Aventis Pharma); diclofenac, 2-[2-(2,6-dichlorophenyl)aminophenyl]acetic acid (Biogalenica).
[b] With their respective 95% confidence limits.
[c] Maximal inhibition.

Compounds **1** and **3**, isolated from an acetonic extract, caused potent antinociceptive effects in the same experimental model with calculated ID_{50} values of 3.9 mg/kg (10.8 μmol/kg) and 1.3 mg/kg (2.5 μmol/kg) (Fig. 4), respectively, both being much more effective than the acetonic extract itself. Comparison with several reference drugs, such as aspirin, paracetamol, diclofenac and dipyrone, indicated that both compounds, but especially compound **3**, were 15- to 65-times more potent than these standard drugs (Table II).

Compound **4**, quercitrin, obtained from the acetonic extract, was not studied here because the previous studies carried out by our laboratories are already published (Meyre Silva *et al.*, 2001; Gadotti *et al.*, 2005).

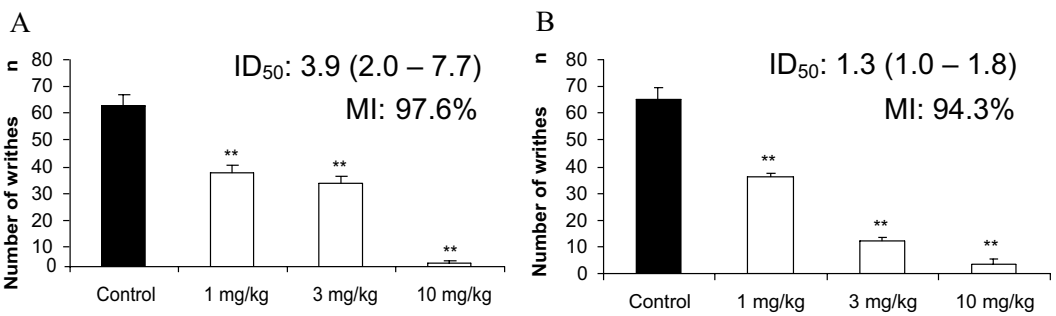


Fig. 4. Antinociceptive effects of (A) compound **1** and (B) compound **3** from *P. glomerata* injected intraperitoneally against writhing in mice. Each group represents the mean of 8 to 10 experiments and the vertical bars indicate the s.e.m. * $P < 0.05$; ** $P < 0.01$.

Although the methanolic extract exhibited potent antinociceptive activity, the phytochemical analyses did not permit the isolation and identification of its components. However, the preliminary results suggest that phenolic compounds are the major constituents. Studies are now in progress to investigate the antinociceptive effects of compounds **1** and **3** in other pharmacological models of pain and also to determine the active principles of the methanolic extract.

In summary, our results demonstrate for the first time the antinociceptive properties of *P. glomerata* as well as the presence of two rare flavellagic acid derivatives with a potent antinociceptive profile.

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