Isolation and HPLC Quantitative Analysis of Antioxidant Flavonoids from *Alternanthera tenella* Colla

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Phytochemical analysis of the antioxidant ethanolic extract of *Alternanthera tenella* Colla led to the isolation of six flavonoids, acacetin 8-*C*-[α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranoside] (**1**), 2″-*O*-α-L-rhamnopyranosyl-vitexin (**2**), 2″-*O*-β-D-glucopyranosyl-vitexin (**3**), vitexin (**4**), quercetin (**5**) and kaempferol (**6**). All the structures were established by ESI-MS and NMR spectroscopic methods. Antioxidant capacity of extract, fractions and isolated compounds was determined using the oxygen radical absorbance capacity (ORAC) assay and extract, fractions and flavonoids isolated showed antioxidant activity *in vitro*. Moreover, the total soluble phenolic contents of the extract and fractions were measured using the Folin-Ciocalteau reagent and the quantitative analysis of flavone C-glycosides major constituents was performed by HPLC.

Key words: Alternanthera tenella Colla, Flavonoids, ORAC Assay

Introduction

The family Amaranthaceae comprises many species, which are used in traditional folk medicine for the treatment of several diseases such as infections, inflammation and fever (Salvador *et al.*, 2002, 2004; Cai *et al.*, 2003; Gorinstein *et al.*, 1991; Siqueira, 1987). Members of this plant family are used in the extraction of natural pigments such as betaxanthin and betalains for application as food colorants and antioxidants (Cai *et al.*, 2003). The Amaranthaceae family includes 65 genera and 1000 species of annual and perennial herbaceous plants, shrubs and some trees occurring in tropical, subtropical and temperate zones of Africa, South America and South East Asia (Siqueira, 1994/1995). Previous chemical analyses have demonstrated the occurrence of anthraquinones, aurone, betacyanins, betalains, betaxanthins, chromoalkaloids, ecdysteroids, flavonoids, protoalkaloids, saponins, steroids and triterpenes (Ferreira *et al.*, 2004; Salvador and Dias, 2004; Brochado *et al.*, 2003; Zhou *et al.*, 1988).

The genus *Alternanthera* Forkssal includes 80 species of which 30 occur in Brazil (Siqueira, 1994/1995). Many species of *Alternanthera* traditionally are used in the treatment of infections, as analgesic, anti-nociceptive, antiviral, antibacterial, antifungal and diuretic agents. Only 16 species have previously been studied regarding their chemical composition. For example, in *Alternanthera* Forkssal the occurrence of flavonoids, isoflavonoids, and flavone C-glycosides has been documented previously (Salvador and Dias, 2004; Salvador *et al.*, 2004; Brochado *et al.*, 2003; Zhou *et al.*, 1988) as well as the occurrence of betacyanins, saponins, steroids, and triterpenes (Salvador and Dias, 2004).

Alternanthera tenella Colla, a herbaceous plant is frequently found in Brazil. In folk medicine, *A. tenella* has been used for the treatment of infections and as diuretic (Salvador *et al.*, 2004; Moraes

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et al., 1994; Siqueira and Guimarães, 1984). Several studies reported anti-inflammatory and immunomodulatory properties of *A. tenella* extracts (Guerra *et al.*, 2003; Moraes *et al.*, 1994). No phytochemical and antioxidant evaluation has previously been reported for *A. tenella*.

Preliminary evaluations of ethanolic extracts of *A. tenella* revealed high levels of polyphenolic compounds as well as a high antioxidant activity. Several studies have reported that specific polyphenols are able to scavenge superoxide and hydroxyl radicals, to reduce lipid peroxyl radicals, and to inhibit lipid peroxidation, and are involved in pathogenesis of various diseases including inflammatory conditions and infections. Plants as sources of antioxidants can be used for medicinal purposes and most of the antioxidant capacity of plants may be due to their polyphenols possessing wide biological properties (Aquino *et al.*, 2001; Cao *et al.*, 1997; Rice-Evans *et al.*, 1996). This prompted us to investigate the phenolic profile and antioxidant capacity of *A. tenella* in more detail. In this study, the ethanolic extract of *A. tenella* was fractionated and six isolated flavonoids were obtained. The isolation and structure identification of major polyphenolic constituents of the ethanolic extract of *A. tenella* were performed and the antioxidant capacity of the extract, fractions and isolated flavonoids was investigated. Moreover, the total soluble phenolic content of the extract and fractions was determined, and the quantitative analysis of flavone C-glycosides was obtained by an HPLC analytical method.

Material and Methods

General experimental procedures

The ^{1}H, ^{13}C NMR and 2D NMR spectra were recorded in DMSO-d_6 on a Bruker Avance DRX spectrometer operating at 400 and 500 MHz for ^{1}H and 100 and 125 MHz for ^{13}C NMR. ESI-MS was performed using a Micromass Quattro LC system both in the positive (30 V) and negative (30 V) mode. IR spectra were obtained in KBr pellets with a Perkin Elmer model 1420 spectrophotometer and UV spectra were recorded on a Hitachi U-3501 spectrophotometer. HPLC separations were carried out on a LC-6A Shimadzu liquid chromatograph equipped with a 3501 UV detector operating at 280 nm and using a reverse-phase separation procedure. The columns Shim-pack ODS (C-18, 4.6 × 250 mm) and Shim-pack ODS (C-18, 20 × 250 mm) were used for analytical and preparative procedures, respectively. Quantitative analyses were performed in a Shimadzu LC-2010C HPLC system, the UV detector was set at 330 nm; 20 μL of each sample was injected into the RP-18 (Lichrospher®, Merck KGaA, Darmstadt, Germany) column (5 μm, 225 × 4.6 mm i.d.) protected by a RP-18 guard column (Merck, 5 μm, 4.0 × 3.0 mm i.d.). Peak areas were calculated by a Shimadzu CLASS-VP™ 7.2.1 integrator. The ORAC assay analyses were carried out on a Synergy HT multi-detection microplate reader system (Biotek, Winooski, VT) in microplates (96 wells). Fluorescence filters with an excitation wavelength of 485 nm and an emission wavelength of 528 nm were used. Total phenolics were analyzed by a microplate reader. 2,2′-Azobis (2-amidinopropane) dihydrochloride (AAPH), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) and fluorescein were obtained from Aldrich (Milwaukee, WI). Caffeic acid, chlorogenic acid, naringenin, quercetin and isoquercitrin were obtained from Carl Roth (Karlsruhe, Germany) or Sigma-Aldrich (St. Louis, MO). Folin-Ciocalteau phenol reagent, sodium carbonate and gallic acid were all purchased from Sigma-Aldrich.

Plant material

Whole plants of *Alternanthera tenella* Colla (Amaranthaceae) were collected at Alto da Boa Vista, Ribeirão Preto, SP, Brazil, in May 2002, and identified by Professor Dr. Josafá Carlos de Siqueira (Pontifícia Universidade Católica, Rio de Janeiro, RJ, Brasil). A voucher specimen is deposited at the Herbarium of the Departamento de Biologia, Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto, Universidade de São Paulo, SP, Brasil (register number SPFR 02968).

Extraction and isolation of compounds

The powdered, air-dried whole plant (1000 g) was extracted exhaustively by maceration at room temperature with hexane and ethanol successively. The spent biomass was filtered from the extracts and the solvents were removed under vacuum in a rotary evaporator (below 40 °C), to obtain the hexanic (yield 13 g) and ethanolic (yield 54 g) crude extracts.

The ethanolic extract (50 g) was suspended in methanol/water (9:1, v/v) and partitioned with hexane (yield 6 g) and dichloromethane (yield

4 g). The alcoholic phase was submitted to an amberlite XAD-2 column eluted with water and ethanol. The ethanolic phase (8 g) was partitioned with *n*-butanol and water to afford an *n*-butanol-soluble portion (2 g). The butanolic phase was chromatographed over a 100 × 5 cm Sephadex LH-20 column using MeOH as eluent with a flow rate of 0.5 mL/min. Subsequently, 110 collected fractions (10 mL each) were monitored by TLC (silica gel; *n*-BuOH/AcOH/H_2O, 65:15:25; $CHCl_3$/MeOH/H_2O, 70:30:3), grouped in 4 major fractions in accordance to similar R_f values, and purified by HPLC on a Shim-pack ODS column (C-18, 20 × 25 mm) at a flow rate of 9.5 mL/min. Fraction I (200 mg) was purified using MeOH/H_2O (35:65, v/v) as the eluent to yield pure compounds **1** (30 mg, t_R = 15 min), **2** (20 mg, t_R = 8 min) and **3** (3 mg, t_R = 10 min). Fraction II (100 mg) was purified using MeOH/H_2O (40:60, v/v) to yield pure compounds **1** (10 mg, t_R = 18 min), **2** (60 mg, t_R = 12 min) and **4** (4 mg, t_R = 10 min). Fraction III (220 mg) was purified using MeOH/H_2O (45:65, v/v) to yield pure compounds **1** (10 mg, t_R = 22 min), **2** (100 mg, t_R = 16 min), **3** (2 mg, t_R = 18 min) and **4** (5 mg, t_R = 14 min). Finally fraction IV (30 mg), containing flavonoids aglycone, was purified with 70:30 MeOH/H_2O to yield compounds **5** (5 mg, t_R = 8 min) and **6** (6 mg, t_R = 10 min).

Acacetin 8-C-[α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranoside] (**1**): Amorphous powder; m.p. 208–210 °C. – $[\alpha]_D^{25}$ = – 42.4° (MeOH, *c* 0.1). – UV: λ_{max} (MeOH) = 338, 278, 254 nm; λ_{max} (NaOH) = 377, 280 nm; λ_{max} ($AlCl_3$) = 341, 304, 278 nm; λ_{max} ($AlCl_3$ + HCl) = 341, 304, 278 nm; λ_{max} (NaOAc) = 377, 280 nm; λ_{max} (NaOAc + H_3BO_3) = 341, 278 nm. – IR (KBr): ν = 3380 (-OH), 2900 (C-H), 1650 (C=O), 1596 (C=C) cm^{-1}. – ^1H NMR and ^{13}C NMR: data were consistent with those previously reported (Sharaf *et al.*, 1997; Harborne, 1996; Markam and Geiger, 1994; Agrawal, 1989; Matsubara *et al.*, 1984). – ESI-MS: *m/z* = 591 $[M-H]^-$, 593 $[M+H]^+$, 615 $[M+Na]^+$.

2″-O-α-L-Rhamnopyranosyl-vitexin (**2**): Amorphous powder. – UV: λ_{max} (MeOH) = 333, 270 nm; λ_{max} (NaOH) = 395, 278 nm; λ_{max} ($AlCl_3$) = 347, 304, 278 nm; λ_{max} ($AlCl_3$ + HCl) = 347, 304, 278 nm; λ_{max} (NaOAc) = 395, 278 nm; λ_{max} (NaOAc + H_3BO_3) = 333, 270 nm. – ^1H NMR and ^{13}C NMR: data were consistent with those previously reported (Harborne, 1996; Markam and Geiger, 1994; Agrawal, 1989). – ESI-MS: *m/z* = 577 $[M-H]^-$, 579 $[M+H]^+$, 601 $[M+Na]^+$.

2″-O-β-D-Glucopyranosyl-vitexin (**3**): Amorphous powder. – UV: λ_{max} (MeOH) = 332, 268 nm; λ_{max} (NaOH) = 395, 324, 281 nm; λ_{max} ($AlCl_3$) = 342, 305, 277 nm; λ_{max} ($AlCl_3$ + HCl) = 342, 305, 277 nm; λ_{max} (NaOAc) = 390, 281 nm; λ_{max} (NaOAc + H_3BO_3) = 342, 277 nm. – ^1H NMR and ^{13}C NMR: data were consistent with those previously reported (Harborne, 1996; Markam and Geiger, 1994; Agrawal, 1989). – ESI-MS: *m/z* = 593 $[M-H]^-$, 595 $[M+H]^+$, 617 $[M+Na]^+$.

Vitexin (**4**): Amorphous powder. – UV: λ_{max} (MeOH) = 334, 270 nm; λ_{max} (NaOH) = 395, 280 nm; λ_{max} ($AlCl_3$) = 342, 305, 278 nm; λ_{max} ($AlCl_3$ + HCl) = 342, 304, 278 nm; λ_{max} (NaOAc) = 395, 280 nm; λ_{max} (NaOAc + H_3BO_3) 343, 277 nm. – ^1H NMR and ^{13}C NMR: data were consistent with those previously reported (Harborne, 1996; Markam and Geiger, 1994; Agrawal, 1989). – ESI-MS: *m/z* = 431 $[M-H]^-$, 433 $[M+H]^+$, 455 $[M+Na]^+$.

Quercetin (**5**): ^1H NMR and ^{13}C NMR: data were consistent with those previously reported (Harborne, 1996; Markam and Geiger, 1994; Agrawal, 1989). – ESI-MS: *m/z* = 301 $[M-H]^-$.

Kaempferol (**6**): ^1H NMR and ^{13}C NMR: data were consistent with those previously reported (Harborne, 1996; Markam and Geiger, 1994; Agrawal, 1989). – ESI-MS: *m/z* = 285 $[M-H]^-$.

Quantitative HPLC analysis

In order to prepare standard solutions, compounds **1**, **2**, **3** and **4** were dissolved in methanol/water (1:1, v/v). Serial concentrations of these four flavone C-glycosides were prepared at eight concentrations, 0.25–10.00 μg/mL. The flavanone naringenin (at 25 μg/mL) was used as internal standard. Quantitative HPLC analyses were conducted using a RP-18 column (Lichrospher®, 5 μm, 225 × 4.6 mm, Merck). The mobile phase consisted of a linear gradient combining solvent A (acetonitrile) and solvent B (water/acetic acid, 99:1, v/v, pH 2.88) as follows: 15% A (15 min), 15–20% A (7 min), 20% A (5 min), 20–40% A (5 min), 40% A (5 min), 40–15% A (3 min). The analyses were carried out in triplicate at a flow rate of 0.8 mL/min with the UV detector set at λ = 330 nm and an injection volume of 20 μL. Calibration graphs were plotted showing a linear relationship between concentrations versus peak areas for all ref-

erence compounds. The regression equations were $y = 0.0587x - 0.0057$ ($R = 0.9999$) for **1**, $y = 0.0443x - 0.0039$ ($R = 0.9999$) for **2**, $y = 0.0626x - 0.0063$ ($R = 0.9991$) for **3** and $y = 0.0664x - 0.0076$ ($R = 0.9999$) for **4**, where y is the peak area ratio (peak area of reference compounds/peak area of internal standard) and x is the concentration used. Accurately weighed amounts of the ethanolic extract of *A. tenella* and fractions I–IV were dissolved in methanol/water (1:1) and analyzed at the same chromatographic conditions as used for compounds **1**, **2**, **3** and **4**. The attribution of the chromatographic peak was based on the retention times of the single compounds and confirmed by analysis in comparison with the isolated standards. Under our working conditions, the mean of retention time for compounds **1**, **2**, **3**, **4** and naringenin was 12.46, 15.34, 15.87, 28.26 and 35.39 min, respectively. The concentrations of each compound were calculated from the experimental peak areas by analytical interpolation in standard calibration lines. The limit of quantification (LOQ) for all compounds analyzed was 0.3125 μg/mL. Relative standard deviations (%RSD) were in the range of 8.81 and 0.15% calculated as mean of the three replications, whereas for retention times the standard deviations were less than 1%.

Quantitative determination of total soluble phenols

The *A. tenella* dried ethanolic extract and fractions I–IV, dissolved in methanol, were analyzed for their total soluble phenolic content according to the Folin-Ciocalteau colorimetric method (Piccinelli *et al.*, 2004; Aquino *et al.*, 2001). Total soluble phenols were expressed as milligrams of gallic acid equivalents (GAE) per gram of extract or fraction (mg of GAE/g). The analyses were performed in triplicate.

Evaluation of antioxidant capacity by ORAC assay

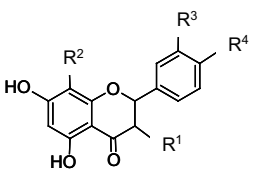
The antioxidant capacity of the ethanolic extract, fractions I–IV and isolated flavone C-glycosides **1**–**4** was assessed through the oxygen radical absorbance capacity (ORAC) assay. The ORAC assay measures the antioxidant scavenging activity against peroxyl radicals using fluorescein as the fluorescent probe. ORAC assays were carried out on a Synergy HT multi-detection microplate reader system. The temperature of the incubator was set at 37 °C. The procedure was carried out according to the method established by Ou and co-workers (2001) with modifications. The data were expressed as micromoles of Trolox equivalents (TE) per gram of extract or fraction on dry basis (μmol of TE/g) and as relative Trolox equivalent for pure compounds. The analyses were performed in triplicate.

Statistical analysis

Data are reported as mean (%RSD, relative standard deviation) of triplicate determinations. The statistical analyses were carried out using the Microsoft Excel 2002 software package (Microsoft Corp., Redmond, WA).

Results and Discussion

The antioxidant ethanolic extract of *A. tenella* was partitioned between *n*-butanol and water and the butanolic phase was fractionated by a Sephadex LH-20 column in order to investigate its chemical constituents. Further purifications of the fractions by reversed-phase HPLC yielded six purified compounds (Fig. 1): acacetin 8-*C*-[α-L-rhamnopy-



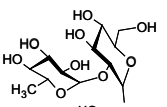
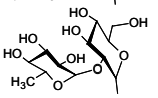
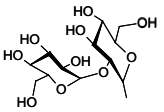
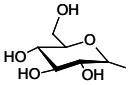
	R1	R2	R3	R4
1	H		H	OCH_3
2	H		H	OH
3	H		H	OH
4	H		H	OH
5	OH	H	OH	OH
6	OH	H	H	OH

Fig. 1. Structures of the compounds acacetin 8-*C*-[α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranoside] (**1**), 2″-*O*-α-L-rhamnopyranosyl-vitexin (**2**), 2″-*O*-β-D-glucopyranosyl-vitexin (**3**), vitexin (**4**), quercetin (**5**) and kaempferol (**6**) isolated from *Alternanthera tenella* Colla.

Table I. Flavone C-glycosides **1**–**4** content of the ethanolic extract and fractions I–IV from *Alternanthera tenella* Colla.

Sample	Flavone C-glycosides content[a]			
	1	**2**	**3**	**4**
Ethanolic extract	1.602 (0.15)	33.04 (1.31)	0.954 (0.15)	2.373 (0.50)
Fraction I	17.05 (1.20)	191.8 (1.80)	16.15 (0.92)	209.1 (2.90)
Fraction II	59.00 (0.99)	756.8 (8.81)	26.90 (1.26)	157.10 (1.21)
Fraction III	56.30 (2.03)	848.2 (5.35)	28.75 (0.88)	65.08 (1.25)
Fraction IV	2.881 (0.59)	51.20 (1.15)	1.851 (0.10)	4.651 (0.67)

[a] Flavone C-glycosides content (mean of triplicate analysis) expressed as mg/g of dried extract or fraction (%RSD, relative standard deviation).

ranosyl-(1→2)-β-D-glucopyranoside] (**1**), 2″-*O*-α-L-rhamnopyranosyl-vitexin (**2**), 2″-*O*-β-D-glucopyranosyl-vitexin (**3**), vitexin (**4**), quercetin (**5**) and kaempferol (**6**). The isolated compounds were identified comparing their physical and spectroscopic properties including 1D (^{13}C, DEPT and ^{1}H) and 2D NMR (TOCSY, HMQC and HMBC), ESI-MS, IR and UV spectra with those reported in the literature (Aquino *et al.*, 2001; Sharaf *et al.*, 1997; Harborne, 1996; Markam and Geiger, 1994; Agrawal, 1989; Matsubara *et al.*, 1984). The occurrence of flavone C-glycosides in *A. tenella* shows chemotaxonomic significance and reinforces the data presented in the literature, once characterized this species as being chemically in accordance with other species of the genus *Alternanthera* (Salvador and Dias, 2004; Zhou *et al.*, 1988).

The HPLC analysis revealed that flavone C-glycosides **1**, **2**, **3** and **4** were the major constituents of the analyzed samples. The HPLC quantitative method was validated (ICH. Q2B, 1996) and the content of these compounds in the ethanolic extract and in the fractions I, II, III and IV are shown in Table I. The limit of quantification (LOQ) for all compounds analyzed was 0.3125 μg/mL and the relative standard deviations (%RSD) were in the range of 8.81 and 0.15%.

Natural antioxidants, such as flavonoids and other phenolic compounds of plants, may have one or more of the following functions: free radical scavengers; enzyme inhibitory actions; protection against lipid peroxidation and quenchers of reactive oxygen species, such as superoxide anion (Cao *et al.*, 1997; Rice-Evans *et al.*, 1996).

The findings obtained in this study demonstrate that the ethanolic extract of *A. tenella* possessed antioxidant/free-radical scavenging effectiveness, which seems to be correlated to its total phenolic content. The antioxidant activity of the complex phenolic pool contained in the ethanolic extract of *A. tenella* was lower when compared to the fractions I–IV. The fractions showed a higher total phenols level in correlation to their antioxidant capacity (Table II). The extract appeared to contain a series of C-glycosyl flavones, apigenin derivatives, as major constituents. ORAC assay results for flavones **1**–**4** and results for quercetin, isoquercitrin, chlorogenic acid and caffeic acid (reference compounds) are summarized in Table III. All of the compounds assayed demonstrated a radical scavenging activity, which was lower than that of quercetin. The values indicate that the number of free phenolic -OH groups and small structural differences of the assayed compounds potentially could contribute significantly to differences in the magnitude of the ORAC assay value. For example, the presence of an -OMe group in ring B of compound **1** in substitution to a -OH group in compound **2** decreased the antioxidant capacity. Re-

Table II. Total phenols content and antioxidant capacity of the ethanolic extract and fractions I–IV from *Alternanthera tenella* Colla.

Sample	Phenol content[a] [mg of GAE/g of extract or fraction][b]	ORAC assay[a] [μmol of TE/g][c]
Ethanolic extract	216.36 (3.62)	848.24 (2.36)
Fraction I	290.61 (0.74)	4542.7 (5.67)
Fraction II	272.58 (0.29)	8416.6 (6.65)
Fraction III	289.09 (0.98)	4690.2 (3.63)
Fraction IV	198.64 (1.81)	1007.2 (1.41)

[a] Mean (%RSD, relative standard deviation) of triplicate assays.
[b] Total phenolics data expressed as milligrams of gallic acid equivalents per gram.
[c] ORAC data expressed as micromoles of Trolox equivalents per gram.

Table III. Antioxidative capacity of flavone C-glycosides **1**–**4** from *Alternanthera tenella* Colla.

Compound	ORAC assay[a]
1	0.72 (1.82)
2	1.41 (1.08)
3	1.92 (1.25)
4	0.96 (1.38)
Quercetin[b]	5.62 (0.89)
Isoquercitrin[b]	5.21 (1.60)
Caffeic acid[b]	2.90 (2.19)
Chlorogenic acid[b]	2.60 (1.84)

[a] ORAC data expressed as relative Trolox equivalent, mean (%RSD, relative standard deviation) of triplicate assays.
[b] Positive controls.

sults from this study are in agreement with data described in the literature (Piccinelli *et al.*, 2004; Aquino *et al.*, 2001; Cao *et al.*, 1997; Rice-Evans *et al.*, 1996).

Thus, the results of this study suggest that the antioxidant capacity of *A. tenella* seems to be correlated to the content of flavonoids, including vitexin derivatives and quercetin, which are present in the ethanolic crude extract. However, further investigations are necessary to confirm if the potential of this plant and flavonoids constituents as a source of antioxidant products power offers a real and useful *in vivo* improvement of the antioxidant status.

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