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Artigo

Brazilian Bromeliaceae species: isolation of arylpropanoid acid derivatives and antiradical potential

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RESUMO: "Espécies de Bromeliaceae brasileiras: isolamento de derivados arilpropanoídicos e potencial antiradicalar". Um total de vinte extratos de seis espécies de Bromeliaceae brasileiras foram avaliadas quanto a atividade antioxidante, usando-se o método colorimétrico de redução do radical DPPH. De maneira geral, os extratos polares dos rizomas das espécies de Bromeliaceae testadas revelaram melhor perfil antioxidante do que os extratos das folhas e dos frutos das mesmas espécies. Os melhores resultados foram encontrados para os rizomas de *Vriesea procera* (Mart. ex Schult.f.) Wittm. e *Neoregelia cruenta* (Graham) L.B. Sm. O extrato bruto metanólico das folhas de *Ananas bracteatus* (Lindl.) Schult. & Schult. f. apresentou uma atividade significativa, em relação aos extratos de folhas testados, e o processo de purificação desse extrato resultou na identificação de quatro metabólitos: ferulato de 2-glicerila, *p*-cumarato de 2-glicerila, 5,7,4'-triidroxi-3,3',5'-trimetoxiflavona e 3-*O*-β-D-glicopiranosil sitosterol.

Unitermos: Bromeliaceae, antioxidante, ferulato de 2-glicerila, p-cumarato de 2-glicerila, 5,7,4'-triidroxi-3,3',5'-trimetoxiflavona.

ABSTRACT: A total of twenty different extracts from six Brazilian Bromeliaceae species was screened for antioxidant activity by assessment of their capacity to scavenge the DPPH radical. In a general way, the polar rhizome extracts from Bromeliaceae representatives showed better antioxidant results than the extracts from leaves and fruits of the same species. The best results were found for the rhizome extracts of *Vriesea procera* (Mart. ex Schult.f.) Wittm. and *Neoregelia cruenta* (Graham) L.B. Sm. Crude methanol extract of *Ananas bracteatus* (Lindl.) Schult. & Schult. f. leaf had a significant antiradical activity among the leaves extracts assessed its purification afforded four metabolites: 2-*O*-feruloyl glyceride, 2-*O*-*p*-coumaroyl glyceride, 5,7,4'-trihydroxy-3,3',5'-trimethoxyflavone and 3-*O*-β-D-glucopyranosyl sitosterol.

Keywords: Bromeliaceae, antioxidant, 2-*O*-feruloyl glyceride, 2-*O*-*p*-coumaroyl glyceride, 5,7,4'-trihydroxy-3,3',5'-trimethoxyflavone.

INTRODUCTION

The family Bromeliaceae fascinates by the exuberance, diversity and beauty of its species. Many of these plants have economical importance as *Ananas comosus*, commonly known as pineapple (Reitz, 1963). Chemical profile of the family is special among the monocotyledons. Several flavonoid compounds (Lewis & Mabrry, 1972; Williams, 1978; Williams & Harbone, 1988; Yano, 1998,2003), anthocyanins (Saito & Harbone, 1983; Scogin, 1985), esters of arylpropanoid acid derivatives (Sutherland & Gortner, 1959; Takata & Scheuer, 1976; Rocha, 1999) have been related to Bromeliaceae species. These types of compounds are known as possessing antioxidant and antiradical activities (Graf, 1992; Cotelle et al., 1996; Rice-Evans et al., 1996, Pietta, 2000; Silva et al., 2000). Antioxidant compounds protect the biological system against the potentially harmful effect of processes or reactions that cause excessive oxidation, involving reactive oxygen (and nitrogen) species (RONS). Excessive formation of RONS has been implicated in several deleterious processes into human organisms such as

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inflammation, arteriosclerosis, diabetes mellitus, arthritis, and cancer and, antioxidants may play an important role in those diseases prevention and therapy (Sies, 1991, 1993; Rocha et al., 2006). Due to all these reasons, the interest in development of new free radical scavengers has been increased. Thus, in this study, crude extracts and semi purified fractions of six Bromeliaceae species *Ananas bracteatus* (Lindl.) Schult. & Schult. f., *Bromelia antiacantha* Bertol., *Alcantarea brasiliana* (L.B. Sm.) J.R. Grant, *Neoregelia cruenta* (Graham) L.B. Sm., *Pitcairnia flammea* Lindl. and *Vriesea procera* (Mart. ex Schult.f.) Wittm. (different parts: rhizomes, leaves, and fruits) were evaluated in order to detect free radical scavenging activities and search the compounds responsible for that activity. Antioxidant activity was assessed using the DPPH (1,1-diphenyl-2-picryl-hydrazyl-hydrate) radical photometric assay in a process guided by its discoloration (Blois, 1958). The antioxidant activity of these extracts was compared with standard solutions of rutin and *Ginkgo biloba*. In this paper, it is also described the isolation of 2-*O*-feruloyl glyceride (**1**) and 2-*O*-*p*-coumaroyl glyceride (**2**), 3-*O*-β-D-glucopyranosyl sitosterol, and 5,7,4'-trihydroxy-3,3',5'-trimethoxyflavone (**3**) from *A. bracteatus* leaves extract.

MATERIAL AND METHODS

General

Comercial rutin was purchased from Merck® and *Ginkgo biloba* extract (EGb 761) used was Tanakan® 40 mg oral solution. DPPH was purchased from Sigma. All reagents, including solvents, used were of analytical grade and obtained from Merck and Vetec. DPPH assay recordings were made in a UV-VIS Spectrometer Shimadzu UV-2200. MS was recorded using a direct inlet system at 70 eV in VG AUTOSPEC equipment. ^{1}H e ^{13}C NMR spectra were run in pyridine-d5 and CD_3OD at 300 and 75 MHz, using Varian Gemini 200 RT UNITYplus-300 Inoval equipment. TLC on silica gel G was visualized using ferric chloride, sulphuric orcinol and vanillin sulphuric.

Plant material

All plants were collected in Brazil and in their natural habitat: *Ananas bracteatus* (Lindl.) Schult. & Schult. f. (318RB) and *Bromelia antiacantha* Bertol. (319RB) were collected at São Pedro do Avaí, Minas Gerais, in 1995; *Alcantarea brasiliana* (L.B. Sm.) J.R. Grant (R196007) and *Pitcairnia flammea* Lindl. (R19005) were collected at Teresópolis, Rio de Janeiro; in 1999, *Neoregelia cruenta* (Graham) L.B. Sm. (R183238) was collected at Maricá, Rio de Janeiro; in 1999, and *Vriesea procera* (Mart. ex Schult.f.) Wittm. (R19006) was collected at Rio das Ostras, Rio de Janeiro, in 1999. Voucher specimens have been deposited at the Herbarium of Museu Nacional, UFRJ and Herbarium of Jardim Botânico do Rio de Janeiro and identified for Dr T. Wendt and Dr A. Costa.

Extraction and isolation

Leaves (3.2 kg) and fruits (1.2 kg) of *Ananas bracteatus* were dried at room temperature, reduced to small particles and then extracted, separately, in sequence with hexane and methanol by maceration. Fresh fruits (5.4 kg) of *Bromelia antiacantha* were reduced to small particles and extracted by maceration in methanol. Leaves of *Alcantarea brasiliana* (3.3 kg), *Neoregelia cruenta* (4.8 kg), *Pitcairnia flammea* (2.4 kg) and *Vriesea procera* (3.8 kg) were dried at room temperature, reduced to small particles and extracted by maceration in sequence with hexane and the methanol. Rhizomes of *Alcantarea brasiliana* (1.4 kg), *Neoregelia cruenta* (7.3 kg), *Pitcairnia flammea* (4.1 kg) and *Vriesea procera* (1.8 kg) were dried at room temperature, reduced to small particles and extracted by maceration in methanol. All hexanic and methanolic extracts were dried under reduced pressure.

The crude methanolic extract of *A. bracteatus* (EMFA) was suspended in H_2O:MeOH solution (6:4) and partitioned with hexane, CH_2Cl_2, EtOAc and BuOH, affording dichloromethane fraction (PDFA), ethyl acetate fraction (PAFA) and buthanol fraction (PBFA). The PAFA was concentrated under reduced pressure affording 3 g of a residue, which was re-dissolved in a $CHCl_3$:MeOH solution (6:1) and submitted to a liquid-liquid extraction procedure successively with 5% sodium bicarbonate (pH = 9) and 5% sodium carbonate (pH = 14) solutions. The neutral organic phase was then washed with distilled H_2O and concentrated under reduced pressure to a syrup (300 mg). The aqueous sodium bicarbonate fraction was acidified with concentrated HCl (pH = 3) and back-extracted with Et_2O (3 x 80 mL). The Et_2O phase was washed with distilled H_2O, dried over Na_2SO_4, filtered and concentrated under reduced pressure. The resulting residue was chromatographed on Sephadex® LH-20 gel with MeOH yielding 20 mg of compound **1**. The neutral organic phase residue (300 mg) was chromatographed on silica gel 60 eluted with mixtures of hexane, toluene, EtOAc and MeOH. The fractions eluted with mixtures of EtOAc:MeOH (30:70) and MeOH (100) afforded, after crystallization from Me_2CO, 42.5 mg of 3-*O*-β-D-glucopyranosyl sitosterol. The dichloromethane fraction (PDFA) residue (12.5 g) was chromatographed on silica gel 60 eluted with mixtures of hexane, EtOAc and MeOH. The fractions eluted with hexane:EtOAc (40:60) were re-chromatographed on Amberlite XAD-2 with MeOH, after solvent evaporation, the resulting residue was chromatographed on Sephadex® LH-20 gel yielding a mixture (100 mg) of **1**, **2** and **3**. This mixture was chromatographed repeated times on Sephadex® LH-20 gel with MeOH, affording the pure compounds **1** (4 mg) and **3** (3 mg).

2-*O*-feruloyl glyceride (**1**). m.p. 112-114 ºC. EI-MS (70 ev): *m/z* 270 $[M+2]^+$ (2), 254 (4), 238 (3), 207 (3), 194 (3), 147 (70), 119 (23); UV/VIS λ_{max}, (MeOH) nm (log ε): 249 (1.78), 300 (1.76), 328 (2.07); ^{1}H (300 MHz, CD_3OD) and ^{13}C (75 MHz, pyridine-d_5) NMR: Table 1.

2-*O*-*p*-coumaroyl glyceride (**2**). GC/MS: *m/z* 238 $[M^{+\bullet}]$ (15), 207 (4), 164 (31), 147 (100), 119 (29), 65 (19); ^{1}H (300 MHz, CD_3OD) and ^{13}C (75 MHz, CD_3OD) NMR: Table 1.

5,7,4'-trihydroxy-3,3',5'-trimethoxyflavone (**3**). m.p. 232-235 ºC. GC/MS: *m/z* 360 $[M^{+\bullet}]$, 345 $[M^+\text{-}CH_3]$, 343 $[M^+\text{-}OH]$, 330 $[M+\text{-}OCH_3]$, 317 [345-C=O]. UV/VIS λ_{max}, (MeOH) nm (log ε): 364 (3.22), 301 (2.15) and 280 (2.71) nm. ^{1}H NMR (300 MHz, CD_3OD, δ, ppm): 6.25 (1H), 6.53 (1H), 6.73 (2H).

Antiradical activity

Quantitative evaluation of radical scavenging abilities of the samples was carried out by DPPH UV spectrophotometric assay. Sample stock solutions (1.0 mg/mL) were diluted to final concentrations of 250, 125, 50, 25, 10 and 5 µg/mL in ethanol. One ml of a 0.3 mM DPPH ethanol solution was added to 2.5 mL of each different solutions and left to react for 30 min at room temperature. After that the absorbance values were measured at 515-517 nm and converted into the percentage antioxidant activity (AA%) using the following formula:

$$AA\% = 100 - \{[(Abs_{sample} - Abs_{blank}) \times 100]/Abs_{control}]\}$$

Ethanol (1.0 mL) plus plant extract solution (2.5 mL) was used as blank. DPPH solution (1.0 mL, 0.3 mM) plus ethanol (2.5 mL) was used as a negative control and as positive controls standard solutions of rutin (100, 50, 25, 10, 5 e 2.5 µg/mL) and *G. biloba* extract (250, 125, 50, 25, 10 and 5 µg/mL) were used. The EC_{50} values were calculated by linear regression of plots where the abscissa represented the concentration of tested plant extracts, and the ordinate, the percent of antioxidant activity. All the experiments were done in triplicate. The results are given as a mean±standard deviation (SD).

Statistical analysis

Comparisons of data among all groups were done by oneway analysis of variance (ANOVA). Statistical significance was assigned when $p<0.05$. Excel and GraphPad-PRISM 4.00 software had been used for statistical analysis.

RESULTS AND DISCUSSION

Antiradical activity

The 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical is a stable chromogen (purple) radical with maximum absorption ranging from 515 to 517 nm, which can readily undergo reduction by an antioxidant leading to its discoloration. Because of the ease and convenience of this reaction it has been used to free radical-scavenging activity assessment of several kinds of samples (Nakamura et al., 1998; Wettasinghe & Shahidi, 2000; Mensor et al., 2001). The DPPH radical-scavenging activity of Bromeliaceae samples, at different concentrations, was expressed as the percentage reduction of the initial DPPH absorption by the test sample. All hexanic extracts assessed did not show any activity on DPPH radical. Fresh fruits crude methanolic extract of *B. antiacantha* did not show any activity as well. As a general rule, it can be postulated that polar rhizome extracts from Bromeliaceae representatives assayed here show better antioxidant results than those from leaves (ANOVA, $p<0,05$). All the samples showed dose-dependent increase in activity. Figure 1 (**A**) shows that the best results were found to rhizome extracts of *V. procera* (EBMRVP) and *N. cruenta* (EBMRNC). Leaf extracts showed weak to moderate activities. The EBMRVP and EBMRNC are good DPPH radical-scavengers with about 90% of DPPH scavenged under the experimental conditions and the first one (EBMRVP, EC_{50} = 24.01±0.115 µg/mL) showed to be significantly better antioxidant than *G. biloba* extract (EC_{50} = 40.72±0.428 µg/mL). Among the methanolic rhizome extracts, the antioxidant activities decrease in the following order: *V. procera* (EBRVP) > *N. cruenta* (EBMRNC) > *A. brasiliana* (EBMRAB) ≈ *P. flammea* (EBMRPF), whose EC_{50} were determined, respectively, as 24.01±0.0,115 µg/mL, 34.40±0.071 µg/mL, 70.41±0.0,685 µg/mL, 72.90±0.220 µg/mL. According to EC_{50} values, among the methanolic leaf extracts, the activities decrease in the order of *V. procera* EBMFVP (57.54±0.302 µg/mL) > A. bracteatus EMFA (64.24±0.663 µg/mL) > *N. cruenta* EBMFNC (109.08±0,673 µg/mL) > *P. flammea* EBMFPF (160.32±0.571 µg/mL) > *A. brasiliana* EBMFAB (188.53±0.361 µg/mL). Since crude methanolic extract of *A. bracteatus* leaves (EMFA) showed a moderate antiradical activity, it was further fractioned, at first by partition into increasing polarities solvents, affording four sub-fractions: hexane (PHFA), dichloromethane (PDFA), ethyl acetate (PAFA) and buthanol (PBFA). These sub-fractions were evaluated by DPPH assay, Figure 1 (B). PHFA and PBFA did not show any activity, but PAFA (EC_{50} = 23.67±0.309 µg/mL) showed a significant activity, stronger then PDFA (EC_{50} = 160.34±0.790 µg/mL) and EMFA.

Phytochemistry of Ananas bracteatus

Purification of the bicarbonate phase of the *A. bracteatus* ethyl acetate fraction (PAFA) yielded compound **1** ($C_{13}H_6O_6$), which gave a positive reaction with ferric chloride on the TLC. The UV spectrum showed signals at λ_{max} 249, 300 and 328 nm indicative of the presence of aromatic ring conjugated with an α,β-

unsatured carbonyl group (Waterman & Mole, 1994). The EIMS showed $[M+2]^+$ ion peak at *m/z* 270 together with other signals, including the one at *m/z* 194 relative to ferulic acid fragment. The ^{1}H NMR spectrum showed signals for aromatic ring at δ 7.05 (*d; J* = 1.8 Hz; H-2), δ 6.94 (*dd; J* = 1.8 and 8.1 Hz, H-6) and δ 6.77 (*d; J* = 8.1 Hz; H-5), suggesting a 1,3,4-trisubstituted ring. The signal relative to methoxyl group was observed at δ 3.38 in the ^{1}H NMR and at δ 56.13 in the ^{13}C NMR spectra. The ^{1}H NMR spectrum also showed the signals for the *trans*-coupled olefinic protons at δ 7.53 (*d; J* = 15.8 Hz; H-7) and δ 6.30 (*d; J* = 15.8 Hz; H-8), which was confirmed by signals in the ^{13}C NMR spectrum at δ 147.60 (C-7) and 115.90 (C-8). The NMR spectral data of **1** (Table 1) were very similar to those published for 1-*O*-feruloyl glyceride (**4**) (Shimomura et al., 1987), except for the glyceryl unit. The ^{13}C NMR spectrum of **1** showed signals at δ 66.30 (OCH_2) and at δ 68.20 (OCH) which were attributed, respectively, to C-1'/C-3' and C-2' of the glyceryl moiety, indicating that ferulic acid was linked to the secondary hydroxyl group of glycerol, similarly 2-*O*-p-coumaroyl glyceride (Dong-Zhe et al., 1996). Analysis of the spectral data and comparison with literature records (Asakawa et al., 1977; Koshino et al., 1988) allowed proposing for compound 1, the structure of the 2-*O*-feruloyl glyceride. Purification of neutral organic phase afforded a white powder which was identified by comparison with literature records as 3-*O*-β-D-glucopyranosyl sitosterol.

Table 1. ^{13}C- and ^{1}H-NMR spectral data for compounds 2-*O*-feruloyl glyceride (**1**) and 2-*O*-*p*-coumaroyl glyceride (**2**)$^{a,b.}$

C/H	1		2	
	δC (pyridine-d_5)	δH (CD_3OD)	δC (CD_3OD)	δH (CD_3OD)
1	C, 127.03		C; 127.11	
2	CH, 114.96	7.05 (*d, J* = 1.84)	CH; 131.21	7.46 (*d, J* = 8.61)
3	C, 150.38		CH; 116.82	6.78 (*d, J* = 8.61)
4	C, 149.15		C; 161.26	
5	CH, 116.71	6.77 (*d, J* = 8.1)	CH; 116.82	6.78 (*d, J* = 8.61)
6	CH, 122.76	6.94 (*dd, J* = 1.84 and 8.1)	CH; 131.21	7.46 (*d, J* = 8.61)
7	CH, 147.60	7.53 (*d, J* = 15.83)	CH; 146.99	7.66 (*d, J* = 15.93)
8	CH, 115.90	6.30 (*d, J* = 15.83)	CH; 114.77	6.36 (*d, J* = 15.93)
9	C, 167.40		C; 166.09	
1' and 3'	CH2, 66.30	3.80-4.30	CH_2; 66.38	4.06-4.40
2'	CH, 68.20	3.80-4.30	CH; 68.58	4.06-4.40
OCH_3	56.13			

^{a}The chemical shifts to ^{13}C were determined at 75 MHz and the values are in ppm downfield from TMS and multiplicities were obtained from APT experiments. ^{b}The chemical shifts to ^{1}H were determined at 300 MHz. Values are in ppm downfield from TMS and *J* value in Hz in parentheses.

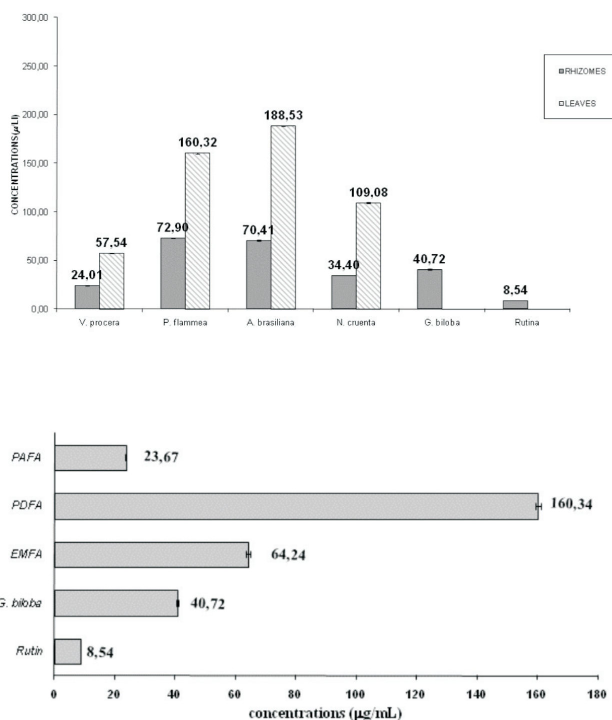
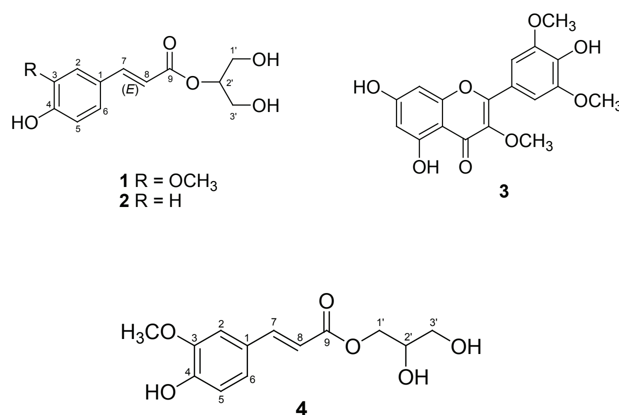


Figure 1. DPPH free radical scavenging activity (EC_{50}) of crude extracts and semi purified-fractions of Bromeliaceae species. (**A**): Comparasion between EC_{50} for the crude methanolic extracts obtained from leaves and rhizomes of Bromeliaceae species. (**B**): Comparison between EC_{50} for fractions dichlorometane (PDFA) and ethyl acetate (PAFA) obtained from the total methanolic extract of leaves (EMFA) of *A. bracteatus*.

From purification of dichloromethane fraction (PDFA), compounds **1, 2** and **3** could be identified. Compound 2 and 3 had their structures determined by comparison of their spectral data with literature records (Dong-Zhe et al., 1996; Yu et al., 1987) as 2-*O*-*p*-coumaroyl glyceride and 5,7,4'-trihydroxy-3,3',5'-trimethoxyflavone, respectively.



Among naturally occurring phenolic compounds, phenolic acids (and their derivatives) and flavonoids are of particular interest because their potential biological properties, such as anti-inflammatory, anti-allergic, antimicrobial, anticarcinogenic, antiviral and antioxidant activities (Tiwari, 2001; Cai et al., 2004). The potent antioxidant activity of many phenolic acid derivatives can probably be attributable to their radical scavenging activity, although other mechanisms may be involved such as formation of metal chelating. It's known that antiradical activity of phenolic compounds depends on their molecular structure which is related to availability of phenolic hydrogens and the possibility of stabilization of the resulting phenoxyl radicals formed by hydrogen donation (Pannala et al., 2001). Ferulic acid, due to its phenolic nucleus and an extended side chain conjugation, quickly forms a resonance stabilized phenox radical which accounts for its potent antioxidant potencial, but ferulic acid can not form a metal chelate (Cotelle et al., 1996). The esterification of the carboxyl group seems to affect the antiradical activity of phenolic acids in a different way. Some studies claim that for phenolic acids owning ethylenic side chain there is an enhance of the antiradical activity, and for those dihydroxyphenolic acid derivatives the esterification led to a dramatic decrease in their scavenging activity (Del Maestro, 1980). The authors suggest that when the carboxyl group of coplanar conformation compounds was esterified, the rotation of the phenyl moiety may have been restrained, leading to conformational modification that enhances antiradical activity. Ferulic acid derivatives are expected to be more effective than p-coumaric acid derivates because the electron-donating methoxy group allows increased stabilization of the resulting aryloxyl radical through electron delocalization after hydrogen donation by the hydroxyl group (Cotelle et al., 1996).

Flavonoids radical scavenging activity depends on the structure and the substituents of the heterocyclic and aromatic rings. Thus, the maximum effectiveness for radical scavenging apparently requires the 3-OH group attached to the 2,3-double bond and adjacent to the 4-carboxyl in the C ring, and even presence of the two hydroxyl group in the ortho-diphenolic arrangement in the B ring that has the better electron donating properties and can chelate trace metals such as iron and copper. Blocking the 3-hydroxyl group in the C-ring of the flavonoids, such as 5,7,4'-trihydroxy-3,3',5'-trimethoxyflavone (**3**) (Figure 1), reduces greatly radical scavenging activity. In the absence of the dihydroxy structure in the B ring there are only contribution to the antioxidant activity from hydroxyl on the A ring (5,7-dihydroxyphenolic arrangement). 5-OH group with 4-oxo function A and C rings contribute for the antiradical activity (binding sites for trace metals).

The antioxidant activity assessments of Bromeliaceae species by measuring their capacity to scavenge the DPPH radical showed that, in a general way, the polar rhizome extracts from Bromeliaceae representatives showed better antioxidant results than leaves and fruits from the same species. The best results were found for the rhizome extracts of *V. procera* and *N. cruenta.* Crude methanolic extract of *A. bracteatus* leaves (EMFA) had a considerable antiradical activity among the leaves extracts assessed and its ethyl acetate fraction (PAFA) showed the best profile being even higher than crude methanolic extract. The purification of this fraction revealed the richness in ferulic acid derivative, which was identified by spectrometric data as 2-*O*-feruloyl glyceride, an uncommon kind of glyceryl compound derivative. Dichloromethane fraction (PDFA) showed comparatively weak activity. From purification of this fraction, three compounds were identified *O*-feruloyl glyceride, 2-*O*-*p*-coumaroyl glyceride and 5,7,4'-trihydroxy-3,3',5'-trimethoxyflavone. The 2-*O*-feruloyl glyceride was more likely to be responsible for most of the observed antiradical activity of the crude methanolic extract of *A. bracteatus* leaves, because of its structure supplies.

This work demonstrates the efficiency of the DPPH assay in the search for new natural compounds with antiradical scavenging properties. Knowledge of the antiradical properties of these samples is worthy of research because it may be a very important basis to their possible biological properties related with deleterious oxidative processes.

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