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ORIGINAL ARTICLE

Antioxidant activity of flavonoids from *Croton sphaerogynus* Baill.

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Abstract Since the early days of medicine, chemical substances derived from animals, plants, and microorganisms have been used for treatment of various diseases. Among these, products derived from plants have dominated the pharmacopoeias for thousands of years, providing an inexhaustible source of medicinal resources. *Croton* L. is the second largest genus of Euphorbiaceae, comprising about 1300 species of trees, bushes, and herbs. *Croton sphaerogynus* Baill. belongs to the same clade of *Croton cajucara* Benth, used in traditional medicine. The aim of this study was to characterize the antioxidant activities of extracts and isolated flavonoids obtained from leaves of *C. sphaerogynus*. Chemical composition of leaf extracts and its fractions (flavonoid contents) and in vitro antioxidant properties were examined. Two flavonoids were isolated: quercetin 3-*O*-methyl ether and kaempferol 3-*O*-methyl ether. Antioxidant activity was higher, especially for fractions composed mainly by quercetin 3-*O*-methyl ether compared to crude extract and other fractions. For those fractions, EC_{50} values were found to be close to the ones presented by quercetin when they were analyzed for their radical scavenging activities.

Keywords Antiradical · Flavonol derivatives · Ion reduction · Metal chelating

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Introduction

Since the early days of medicine, chemical substances derived from animals, plants, and microorganisms have been used for treatment of various diseases (Schmidt et al. 2008; Dewick 2009). Among these, products derived from plants have dominated the pharmacopoeias for thousands of years, providing a source of medicinal resources (Ngo et al. 2013).

Euphorbiaceae is the third largest family of Angiosperms (Koehn and Carter 2005). In many parts of the world, species of this family are known to have medicinal or toxic properties. The second largest genus of Euphorbiaceae is *Croton*, comprising about 1300 species of trees, shrubs, and herbaceous plants. *Croton* species are distributed in tropical and subtropical regions of the new and the old world (Souza and Lorenzi 2005).

In many parts of Asia, Africa, and America, *Croton* species are used in traditional medicine. In Brazil, *Croton* species are used for treatment of a wide variety of ailments, such as hypercholesterolemia, obesity (*C. cajucara* Benth); inflammation, ulcers, leukemia (*C. celtidifolius* Baill.); anorexia (*C. nepetifolius* Baill.); wounds, intestinal inflammation (*C. palanostigma* Klotzsch.); rheumatism, cancer (*C. urucurana* Baill.); anorexia, gastrointestinal disorders, and also as a sweetener (*C. zehntneri* Pax & K. Hoffm.) (Salatino et al. 2007).

Many species of *Croton* produce red latex and are popularly known as "sangre-de-drago." *C. lechleri* Müll. Arg., *C. macrobothrys* Baill., *C. celtidifolius*, *C. palanostigma*, and *C. urucurana* are among the species which produce red latex, all are native in Brazil. Latex composition was described with the presence of proanthocyanidins and some alkaloids, also presenting antibacterial (Chen et al. 1994) and antiproliferatve activity on leukemia cells (Rossi et al. 2003).

There are many studies focusing on the pharmacological effects of extracts and isolated substances produced by different plant parts, such as leaves and stems of *Croton* species. As an example, the water and ethanol extracts of *C. schiedeanus* Schltdl. presented antihypertensive activity (Guerrero et al. 2001, 2002a). Among the compounds isolated from *Croton*, trans-dehydrocrotonin has received increased attention on its pharmacological activity. This alkaloid obtained from *C. cajucara* was confirmed as hypoglycemic and hypolipidemic (Maciel et al. 2002), and anti-estrogenic and anti-tumor agent (Grynberg and Echevarria 1999). Diterpene plaunotol, isolated from *C. sublyratus* Kurz., potentiated the drug activity against *Helicobacter pylori* (Marshall et al. 1985) Goodwin et al. 1989 (which causes peptic ulcer) (Koga 2002) and showed anti-angiogenic activity (Kawai et al. 2005).

Croton is a challenge for phylogenetic studies. In 2011, a molecular phylogeny by van Ee and colleagues focused on the *Croton* species from the New World, dividing the genus into four subgenera (subgenus *C.* subg. *Quadrilobi* (Müll. Arg.) Pax in Engl. & Prantl, *C.* subg. *Adenophylli* (Griseb.) Riina, *C.* subg *Geiseleria* A. Gray, and *C.* subg *Croton* (exclusively of Old World) and describes 10 new sections, resulting in 31 sections that include all species of the New World (van Ee et al. 2011).

Croton section *Cleodora* (Klotzsch) Baill. comprises 18 species distributed in most or seasonally dry forest in tropical South America, Central America, and Mexico (van Ee et al. 2011). Species belonging to this section are commonly used for medicinal preparations by indigenous and traditional communities, in the treatment of wounds and prevention of infection (Salatino et al. 2007). The best-known species of this section is *C. cajucara*, widely distributed in the Amazon basin, and which has an extensive history of ethnobotanical uses (van Ee et al. 2011).

Croton sphaerogynus Baill. (*Croton* sect. *Cleodora*) is widely distributed in Brazilian seashore plains moist forest ("restinga forest") (Caruzo et al. 2013), being native and endemic to Brazil. Taking into account the chemotaxonomic criteria, species belonging to *Croton* section *Cleodora* would present promising bioprospecting potential, since species of this section have been referenced as having bioactive substances.

Croton sphaerogynus had already been described as a major producer of diterpenes (Motta et al. 2013; Santos et al. 2015). Motta et al. (2013) observed moderate antiproliferative activity of the leaf dichloromethane extract (mean Log $GI_{50} = 0.86$), a fraction rich on diterpenes. Santos et al. (2015) used maceration at room temperature to extract plant constituents and observed a similar composition of diterpenes to that described by Motta et al.'s study (2013), which used serial extraction of plant material in hexane, dichloromethane, and methanol. Both studies tested the antiproliferative activities of foliar extract and its fractions, but did not test isolated substances. Moreover, Santos et al. (2015) observed moderate antiproliferative activity of leaf methanol phase, a phase also rich on diterpenes (mean Log $GI_{50} = 0.54$), but when this methanol phase was fractioned, one fraction rich in flavonoids and absent of diterpenes also presented moderate antiproliferative activity (mean Log $GI_{50} = 1.05$), suggesting a possible synergistic combination of flavonoids and diterpenes to enhance the antiproliferative activity of the methanol phase.

Using the same extract described by Santos et al. (2015), the aim of this study was to characterize the antioxidant activity of the crude ethanol extract, their fractions, subfractions and isolated flavonoids from *C. sphaerogynus*, a Brazilian native plant species, in order to better characterize this species and its biological activity.

Materials and methods

Plant material

Croton sphaerogynus Baill. (Figs. 1–3), occurs in the States of Bahia, Rio de Janeiro, Espírito Santo and São Paulo. Most of its populations are from Brazilian seashore plains moist forest ("Restinga forest"). Diagnostic characteristics of this species are the shrub habit; globose, sessile, and brown extra-floral nectaries on the base of leaves; oval-lanceolate to oval leaves, cordate to sagittate, with the basal lobes sometimes overlapping; indumentum of stellate trichomes and inflorescences in spiciform thyrses, with proximal cymules bisexual and distal ones staminate (Caruzo et al. 2013).

A voucher specimen (LBM 65) was deposited in the herbarium Maria Eneyda P. K. Fidalgo (SP), São Paulo. Leaves, and stems of a population of *C. sphaerogynus* were collected in the municipality of Itanhaem, southern coast of State of São Paulo. Samples were dried with aeration at 40 °C for 1 week. Subsequently leaves were grounded for chemical analysis.

Extraction and fractionation

Extraction and fractionation procedures were performed according to Santos et al. (2015). Dried and powdered leaf

Figs. 1–3 Plant material: **1** Adult tree of *C. sphaerogynus*; **2** Female flowers (*double asterisk*) and male flowers (*asterisk*); **3** Glands at the petiole base (photos: Claudia Furlan)

material (1 kg) was extracted by maceration with EtOH for 7 days at room temperature. Crude EtOH extract (EE) was concentrated under reduced pressure, evaporated to dryness under a stream of nitrogen, and lyophilized, affording 70.62 g of crude EtOH extract (yield: 7 %). Part of EE (65 g) was solubilized in MeOH and partitioned using hexane. Hexane phase (HP) was concentrated under reduced pressure to yield 20 g (2 %) of hexane phase, while the MeOH phase (MP) was lyophilized to afford 15 g (yield: 1.5 %), based on dried and powdered leaf material. Part of MP (8 g) was fractionated over Sephadex LH-20 using MeOH as eluent, affording five fractions: F1 (2 g), F2 (0.119 g), F3 (0.090 g), F4 (0.353 g), and F5 (0.090 g). Fraction F4 was fractionated by preparative high-performance liquid chromatography (prep-HPLC1200) using a PrepHT XDB C-18 column (150 × 21.2 mm, 5 µm) affording subfractions F4.1 (1.8 mg) and F4.2 (1.3 mg). Solvent used was 85 % MeOH isocratic for 10 min. Solvent flow was 8 mL min^{-1}, injection volume of 250 µL, and detection at 352 nm.

Hexane phase (HP), already known for its rich presence on diterpenes (Motta et al. 2013; Santos et al. 2015) and its absence on flavonoids, was not used to test the antioxidant activity.

Chemical composition

All lyophilized EE, MP, fractions (F1–F5) and subfractions (F4.1 and F4.2) were dissolved in MeOH (2 mg mL^{-1}) and analyzed by high-performance liquid chromatography (HPLC, 1260 Agilent Technologies) using diode array detector (DAD) and a Zorbax-C18 column (150 × 4.6 mm, 3.5 µm) at 40 °C. Solvents used were 0.1 % acetic acid (AcOH) and acetonitrile (CH_3CN), starting with 15 % of CH_3CN (0–20 min), increasing to 100 % (20–25 min); isocratic (5 min); decreasing to 15 % (30–32 min); isocratic (3 min). Solvent flow rate was 1.5 mL min^{-1} (0–25 min), 1.0 mL min^{-1} (25–26 min), 1.5 mL min^{-1} (26–35 min); injection volume of 3 L, and detection at 352 and 280 nm. Quercetin and kaempferol at concentrations from 0.0006 up to 0.36 µg mL^{-1} were used to prepare calibration curves following the same analysis conditions. Results are expressed as quercetin equivalent milligrams (QE) or kampferol equivalent milligrams (KE) per gram of dry sample (mg g^{-1}).

MP was also submitted to high-performance liquid chromatography coupled mass spectrometry (HPLC–MS); Zorbax SB-C18 (150 × 4.6 mm, 5 µm). Solvents: 0.1 % acetic acid and acetonitrile starting with 15 % CH_3CN (0–20 min), increasing to 100 % (20–25 min), isocratic (5 min), decreasing to 15 % (30–32 min) and isocratic (3 min). Flow 90 µL min^{-1}, voltage 4000 V, nebulizer 27 psi, drying gas at 320 °C and flow of 7 L min^{-1}. Constituents were identified by comparing the corresponding UV–Vis and ESI/MS–MS spectra with MS data from the literature.

For establishment of substitution patterns, F4.1 and F4.2 were submitted to UV–Vis absorption spectroscopy

(240–600 nm) using methanol solution and ionization (KOH, NaOAc) or complexing ($AlCl_3$, $AlCl_3$/HCl, NaOAc/H_3BO_3) shift reagents (Markham 1982). Kaempferol and quercetin were identified by co-chromatography over cellulose thin layer chromatography (TLC) by comparison with authentic samples using BAW (*n*-butanol:acetic acid:water—4:1:5) and CAW (chloroform:acetic acid:water—30:15:2) and UV–Vis analysis (Markham 1982). Identification of sugar presence was carried out with chemical hydrolysis (1 N hydrochloric acid). Hydrolysis products were identified by co-chromatography over cellulose TLC by comparison with authentic samples using 15 % acetic acid.

Free radical scavenging activity determination using DPPH$^{\bullet}$ (2,2-Diphenyl-1-picrylhydrazyl)

Free radical scavenging activities of all samples were determined according to the DPPH radical method modified from Furlan et al. (2015). DPPH solution in methanol (0.2 mM) was freshly prepared and 200 µL were mixed with 20 µL of each sample. Reaction mixture was incubated for 20 min at room temperature and in the dark. The decrease in absorbance was measured at 515 nm by an EPOCH microplate reader. As positive control, Trolox (6–200 µg mL^{-1}) and quercetin (7.5–120 µg mL^{-1}) were used. Methanol was used as negative control.

Free radical scavenging activity determination using ABTS$^{\bullet}$ (2,2′-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)

Scavenging activity of the ABTS radical followed the protocol described by Rufino et al. (2007) with modifications. The ABTS solution (3.84 mg mL^{-1}) was prepared using ultrapure water and a solution of 2.6 mM potassium persulfate. For the formation of ABTS radical, the two solutions above were mixed in a ratio of 1:1. Mixture was maintained in the dark at room temperature for 12–16 h. At the time of the test, 1 mL of the ABTS$^{\bullet}$ previously prepared was diluted in 30 mL of methanol. To each 20 µL of sample and controls were added 280 µL of the diluted solution of ABTS$^{\bullet}$. Microplate was incubated for 2 h at room temperature in the dark. The absorbance was detected at 734 nm by an microplate reader. Trolox (12–250 mg mL^{-1}) and quercetin (15–120 mg mL^{-1}) were used as positive controls. Methanol was used as negative control.

Ferric-reducing antioxidant potential (FRAP)

Ferric-reducing power of samples was determined according to a modified protocol from Furlan et al. (2015). FRAP solution was prepared on the day of analysis by mixing 25 mL of acetate buffer (0.3 M, pH 3.6), 2.5 mL of 10 mM TPTZ and 2.5 mL of 20 mM ferric chloride. FRAP solution (265 µL) was mixed with 10 µL of each sample and 25 µL of ultrapure water. After incubation of 30 min at 37 °C, the absorbance at 595 nm was detected by an microplate reader. Trolox (6–200 mg mL^{-1}) and quercetin (7.2–180 mg mL^{-1}) were used as positive control. Methanol was used as negative control.

β-carotene bleaching assay

Antioxidant activity by β-carotene bleaching assay was determined according to Duarte-Almeida et al. (2006), with modifications. The reactive mixture was prepared in 500 µL of dichloromethane: 21 µL of linoleic acid, 100 mg of Tween 40 and 210 µL of β-carotene solution in dichloromethane (2 mg mL^{-1}). Solvent was evaporated under nitrogen until complete dryness and 32 mL of water previously saturated with oxygen, were vigorously mixed. Absorbance of the solution was measured by a microplate reader to ensure that was between 0.5 and 0.6 at 450 nm. Reaction solution (250 µL) was mixed to 10 µL of samples or standards. First reading was made immediately and the mixture was incubated at 45 °C. Readings were made at intervals of 15 min for 120 min. As positive control, Trolox (6–200 µg mL^{-1}) and quercetin (7.5–120 µg mL^{-1}) were used. Methanol was used as negative control.

Metal chelating activity

Ferric chelating activity was performed according to Min et al. (2011), by mixing 10 µL of each sample or control with 130 µL of 10 % acetate buffer, 10 µL of 1 mM ferrous ammonium sulfate, and 10 µL of 6.1 mM ferrozine solution (after 5 min). Microplate was kept at room temperature for 10 min, and absorbance at 562 nm was detected by an microplate reader. A methanolic solution of gallic acid (5–100 mg mL^{-1}) was used as a positive control and methanol as negative control.

Determination of oxygen radical absorbance capacity (ORAC)

ORAC assay was adapted from the protocols proposed by Min et al. (2011). Sodium fluorescein was dissolved in phosphate buffer solution (PBS) (75 mM, pH 7.0) to obtain a stock solution of 4.0 µM. The fluorescein working solution (8 nM) was freshly prepared in PBS, and 150 µL of this was mixed with 25 µL of each sample (in PBS) at different concentrations. The reaction mixtures were incubated for 30 min at 37 °C, and 25 µL of 75 mM and AAPH (2,2′-Azobis(2-methylpropionamidine) dihydrochloride) solution (152 mM) were added to the wells.

As positive controls, a PBS Trolox solution was plotted (6.25–50 mM). A blank with PBS was run with each assay. The fluorescence (excitation = 485 nm; emission = 520 nm) was registered 120 times with a delay of 60 s between repeats using a microplate reader. The antioxidant capacity was based on the calculation of the area under the curve (AUC), using the following formula:

$$(\mathrm{AUC}) = 1 + f_1/f_0 + f_2/f_0 + f_3/f_0 + \ldots f_i/f_0,$$

where f_0 is the initial fluorescence reading at 0 min, and f_1 is the fluorescence reading at time 1. The net AUC was obtained by subtracting the AUC of the blank from the AUC of the sample. The final ORAC values were calculated by using a regression equation between Trolox concentrations and the net AUC.

For all antioxidant assays, sample antioxidant potential was expressed as milligram per gram of dry extract equivalents to positive control (Trolox, quercetin, or galic acid) and effective concentration of each sample to achieve 50 % of the antioxidant activity (EC_{50} μg mL^{-1}).

Results and discussion

Croton sphaerogynus had already been described as a major producer of diterpenes (Motta et al. 2013; Santos et al. 2015). According to Santos et al. (2015), maceration at room temperature resulted in a similar composition of diterpenes described by Motta et al. (2013). Using the same extract used by Santos et al. (2015), partition using MeOH:Hexane was not efficient to eliminate all diterpenes from methanol phase (MP) and from F1. Column chromatography using Sephadex was efficient to eliminate this class of compounds from fractions F2, F3, F4 and F5. As well as, subfractions F4.1 and F4.2 did not present diterpenes. Studies on diterpenes characterization and their relative amounts on each sample were recently published by Santos et al. (2015). Flavonoids' compositions of EE, MP, F1-F5, F4.1, and F4.2 are listed in Table 1.

Twenty flavonoids were detected, mainly quercetin and kaempferol derivatives, but no flavone was detected. F1, F2, and F3 exhibited similar flavonol composition, with the presence of more than five constituents (Table 2). F4 showed a composition of four compounds: isorhamnetin monoglycoside (compound 9), quercitrin (compound 14), quercetin 3-*O*-methyl ether (compound 17), and kaempferol 3-*O*-methyl ether (compound 19), while F5 was composed by one single flavonoid, quercetin 3-*O*-methyl ether (compound 17) (data also presented by Santos et al. 2015).

Fractions F5, F4.1, and F4.2 were subjected to ionizing and complexing reactions for their structural elucidation. Using Markham (1982), it was possible to further characterize the degree of hydroxylation of the flavonoid molecules: substance 17 as quercetin 3-*O*-methyl ether and substance 19 as kaempferol 3-*O*-methyl ether. In addition, comparison of spectroscopic data to those from the literature (Krenn et al. 2003; Bertelli et al. 2012) allowed the confirmation of compound 17 as 5,7,3′,4′-tetrahydroxy-3-methoxy-flavonol (quercetin 3-*O*-methyl ether).

Leaves of *C. sphaerogynus* using maceration in ethanol as extraction method resulted in 0.06 % of substances equivalent to kaempferol and 0.03 % of substances equivalent to quercetin. *C. sphaerogynus* yielded 0.09 % of flavonoids related to dry mass of leaves. Table 2 shows the content of flavonoids detected in each sample analyzed.

The presence of flavonols in many species of *Croton* had already been reported by several authors: Savietto (2011) studying *C. dichrous* Müll. Arg., *C. erythroxyloides* Baill., *C. myrianthus* Müll. Arg., and *C. splendidus* Mart.; Matos (2011) using *C. betulaster* Müll. Arg., *C. glutinosus* Müll. Arg., *C. pycnocephalus* Müll. Arg., *C. montevidensis* Spreng., *C. hemiargyreus* Müll. Arg., *C. antisyphiliticus* Mart., and *C. grandivelum* Baill.; Athayde (2013) studying *C. echinocarpus* Baill. and *C. vulnerarius* Baill.; and Soares (2013) studying *C. pallidulus* Baill. var. *pallidulus* Baill.. On comparing the flavonoid composition of *C. sphaerogynus* to those presented by the above studies, it can be observed the common presence of rutin and a higher proportion of flavonols instead of flavones. However, unlike *C. dichrous*, *C. erythroxyloides*, *C. myrianthus*, C. *pallidulus* var. *pallidulus*, *C. echinocarpus*, and *C. vulnerarius*, *C. sphaerogynus* did not present the acylated flavonoid tiliroside (kaempferol *p*-coumaroyl).

Furlan et al. (2015), studying nine Argentinian species of *Croton*, identified the presence of tiliroside in all samples, but *Croton* species studied were from sections *Adenophylli*, *Barhamia*, *Lamprocroton*, and *Pedicellati.* This acylated flavonol was also detected by Matos (2011) when investigating species from *Croton* sections *Barhamia*, *Argentini*, and *Cleodora*, being *C. hemiargyreus* (*Croton* sect. *Cleodora*) the species with less expressive amount of this flavonoid. Maciel et al. (2002), studying *C. cajucara*, also from *Croton* sect. *Cleodora*, reported the presence of kaempferol derivatives, mainly kaempferol 3,4,7-thymethyl ether, but the complete absence of tiliroside. Although tiliroside could be a taxonomic marker for *Croton*, these studies suggest differences in flavonoid composition among *Croton* sections.

Flavonoids highly methoxylated were also described as frequent in *Croton* species. For *C. schiedeanus* and *C. cajucara* were reported quercetin 3,7-dimethyl ether, rutesin, artemetin, and methoxylated kaempferol derivatives (Guerrero et al. 2002b; Maciel et al. 2002; Salatino et al. 2007).

Several in vitro methods are used to determine the antioxidant capacity of a substance. Currently used are the

Table 1 Major flavonoids constituents found in *Croton sphaerogynus* leaves, characterized by HPLC–DAD (352 nm) and HPLC–MS (Santos et al. 2015)

Constituent	Rt (min)	UV/Vis (nm)	Mass spectrum		Suggestion
			$[m/z]^+$	$[m/z]^-$	
1	1.75	254, 264 (om), 348			Quercetin derivative
2	2.96	256, 264 (om), 296 (om), 354	757.8; 611.1; 465; 303	755.7	Quercetin triglycoside (2 rhamnose; 1 hexose)
3	3.16	260, 296, 356	741.8; 595.1; 433.1; 286.8		Kaempferol triglycoside (2 rhamnose; 1 hexose)
4	4.05	264, 292, 346	741.8; 595.1; 287		Kaempferol triglycoside (2 rhamnose; 1 hexose)
5	4.30	264, 294, 348	741.9; 595.2; 448.7; 287	739.6	Kaempferol triglycoside (2 rhamnose; 1 hexose)
6	4.99	256, 266 (om), 294 (om), 354	595, 303		Quercetin diglycoside (1 pentose; 1 hexose)
7	5.33	256, 266 (om), 294 (om), 354	661.1; 465	608.9	Rutin
8	6.04	264, 294, 348	595, 286.9	593	Kaempferol diglycoside (1 rhamnose; 1 glucose)
9	6.35	256, 264 (om), 296 (om), 354	479, 316.9		Isorhamnetin monoglycoside (1 hexose)
10	7.30	264, 294, 348	448.9; 286.9		Kaempferol monoglycoside (1 hexose)
11	9.23	264, 294, 346			Kaempferol derivative
12	10.10	256, 268 (om), 356			Quercetin derivative
13	10.72	264, 294, 346			Kaempferol derivative
14	10.97	256, 264 (om), 306 (om), 348	449.0; 302.9	447	Quercitrin
15	20.44	262, 294, 340			Kaempferol derivative
16	22.95	254, 270, 298, 370			Quercetin
17	23.23	256, 266 (om), 298 (om), 356	317, 302	314.8	Quercetin 3-*O*-methyl ether
18	23.55	264, 292, 320, 366			Kaempferol
19	23.73	266, 294, 350			Kaempferol 3-*O*-methyl ether
20	23.84	254, 268 (om), 294 (om), 356			Quercetin derivative

Rt: Average retention time in minutes

ones based on power metal reduction, for example, FRAP, or based on anti-radicalar capacity ($ABTS^{\bullet}$, $DPPH^{\bullet}$), on quantifying products formed during lipid peroxidation (oxidation of β-carotene), and on ORAC (Sanchez-Moreno 2002; Sánchez-Burgos et al. 2013; Roby et al. 2013; Thatoi et al. 2014).

Antioxidant potentials of samples are shown in Table 3. Both tests of radical scavenging capacity ($DPPH^{\bullet}$ and $ABTS^{\bullet}$) yielded similar results with F3, F4, and F5 being the most active samples. F4 was the sample that showed the highest content of ferric-reducing capacity (1000 mg g^{-1} TE) followed by F5 (813.97 mg g^{-1} TE). For metal chelating activity, F4.2 presented the highest chelating potential (229.01 mg g^{-1} GAE) followed by F4 (136.53 mg g^{-1} GAE). For β-carotene bleaching assay, Trolox calibration curves showed no linearity between concentration versus intensity of the response, and hence, values of Trolox equivalent were not calculated. For ORAC, F4.1 presented the highest absorbance capacity (3536.14 μM g^{-1} TE) followed by F4.2 (2976.66 μM g^{-1} TE).

According to Table 3, most fractions presented higher antioxidant activities than ethanolic extract (EE) and methanol phase (MF). This result indicates the antioxidant activities of the samples are strongly related to flavonoid composition. F1 showed lower antioxidant activity compared to F2 and F3, presenting also lower contents of flavonoids. F2 and F3 had relatively similar contents and compositions of flavonols and also presented similar antioxidant capacity. F4 was the most active sample in almost tests (except for metal chelating activity and ORAC), suggesting compound 17 (quercetin 3-*O*-methyl ether) and 19 (kaempferol 3-*O*-methyl ether) as responsible for the antioxidant properties of this fraction. F5, composed exclusively by quercetin 3-*O*-methyl ether showed very similar results to F4, but not higher.

These results suggest that antioxidant capacity seems to be directly related to a synergy between quercetin derivatives in F4. Synergistic interactions among chemical substances may potentiate the effect of an extract, or also can interfere with its action (Lila and Raskin 2005). Besides the composition, contents of those flavonols in both F4 and F5 are probably related to their activities, since F4 has contents of quercetin derivatives approximately twice higher than F5 (Table 2).

F4 was the most active fraction in $DPPH^{\bullet}$, β-carotene, and FRAP assays, and its fractionation was an attempt to isolate compounds 14 and 19 (Table 2) and test their

Table 2 Amount of major flavonoids (mg/g) of *Croton sphaerogynus* crude EtOH extract (EE), MeOH (MP) phase, fractions (F1–F5) and subfractions (F4.1 and F4.2) of *C. sphaerogynus*, detected by HPLC–DAD (352 nm)

Constituent*	EE	MP	F1	F2	F3	F4	F5	F4.1	F4.2
1	nd	0.10[a]	–	0.47[a]	–	–	–	–	–
2	0.20[a]	1.17[a]	0.94[a]	–	–	–	–	–	–
3	nd	–	–	2.23[b]	3.00[b]	–	–	–	–
4	0.57[b]	2.45[b]	2.01[b]	–	–	–	–	–	–
5	1.03[b]	3.73[b]	3.04[b]	–	–	–	–	–	–
6	0.09[a]	0.35[a]	–	4.65[a]	5.18[a]	–	–	–	–
7	0.37[a]	1.63[a]	0.89[a]	19.35[a]	8.39[a]	–	–	–	–
8	0.10[b]	0.51[b]	0.50[b]	–	–	–	–	–	–
9	0.09[a]	0.48[a]	–	0.91[a]	6.51[a]	8.42[a]	–	3,84[a]	–
10	0.18[b]	0.67[b]	0.48[b]	5.5[b]	1.84[b]	–	–	–	–
11	0.99[b]	3.04[b]	2.42[b]	19.71[b]	4.42[b]	–	–	–	–
12	nd	–	–	7.47[a]	8.39[a]	–	–	–	–
13	nd	–	–	4.15[b]	–	–	–	–	–
14	0.51[a]	3.13[a]	–	3.65[a]	34.51[a]	47.06[a]	–	88,09[a]	–
15	nd	–	–	2.54[b]	7.89[b]	–	–	–	–
16	nd	–	–	2.47[a]	3.68[a]	nd	–	4,06[a]	–
17	0.59[a]	3.26[a]	0.13[a]	3.06[a]	2.16[a]	70.96[a]	58.31[a]	14,49[a]	8,06[a]
18	nd	–	–	2.94[b]	1.70[b]	–	–	–	–
19	0.48[b]	0.97[b]	–	–	2.13[b]	29.68[b]	–	–	262,60[b]
20	nd	–	–	–	2.23[a]	nd	–	–	31,35[b]

nd: trace amounts

[a] Values expressed as milligram per gram of quercetin equivalent (mg g^{-1} QE)

[b] Values expressed as milligram per gram of kaempferol equivalent (mg g^{-1} KE)

* Identification suggestions in Table 1

Table 3 Antioxidant capacities of *Croton sphaerogynus* crude EtOH extract (EE), methanol phase (MP), fractions: F1, F2, F3, F4, and F5; and subfractions: F4.1 and F4.2. Radical scavenging activity ($DPPH^{\bullet}$ and $ABTS^{\bullet}$), ferric-reducing antioxidant potential (FRAP), metal chelating activity, and oxygen radical absorbance capacity (ORAC)

Sample	$DPPH^{\bullet}$[a]	$ABTS^{\bullet}$[a]	FRAP[a]	Metal chelating[b]	ORAC[c]
EE	333.71±2.47	311.45±8.06	223.47±7.11	108.15±4.12	761.91±129.40
MP	237.34±2.71	272.51±6.59	184.27±6.49	71.70±8.14	1517.56±267.31
F1	140.00±17.70	173.41±8.38	113.33±1.48	83.54±7.43	1420.44±350.35
F2	450.83±17.74	514.06±7.57	544.89±13.09	68.59±5.34	2872.49±284.09
F3	604.35±9.20	688.19±13.42	765.55±16.09	113.96±4.49	2139.71±318.93
F4	894.33±14.65	961.88±7.34	1000.00±12.27	135.17±8.76	2663.83±252.08
F5	783.60±7.76	849.96±9.93	813.97±9.61	70.15±3.61	2882.54±481.61
F4.1	514.49±6.44	545.70±6.58	595.40±8.02	136.53±0.51	3536.14±621.48
F4.2	80.85±9.48	253.62±6.29	80.80±0.69	229.01±9.16	2976.66±740.27

[a] DPPH, ABTS, and FRAP are expressed as milligrams of Trolox equivalents/gram of dry extract (mg g^{-1} TE)

[b] Metal chelating activities are expressed as milligrams of galic acid equivalents/gram of dry extract (mg g^{-1} GAE)

[c] ORAC activities are expressed as μMol of Trolox equivalents/gram of dry extract (μMol g^{-1} TE)

antioxidant potential when isolated. Analyzing results from F4.2, composed mainly by kaempferol 3-*O*-methyl ether, the antioxidant activity by radical scavenging capacity ($DPPH^{\bullet}$ and $ABTS^{\bullet}$), and ferric-reducing capacity was low when compared to fractions rich on quercetin derivatives. The relationship between function and structure of the flavonoid is the key to the various biological activities performed by those substances. A study performed by van Acker et al. (1996) correlated the structural features of flavonoids with their antioxidant activities and suggested

Table 4 Effective concentration to achieve 50 % of the antioxidant activity (EC_{50}; μg mL^{-1}) of *Croton sphaerogynus* crude EtOH extract (EE), methanol phase (MF), fractions: F1, F2, F3, F4, F5; and subfractions: F4.1 and F4.2. Radical scavenging activities ($DPPH^{\bullet}$ and $ABTS^{\bullet}$), β-carotene bleaching assay, ferric-reducing antioxidant potential (FRAP), metal chelating activity, and oxygen radical absorbance capacity (ORAC)

Sample	$DPPH^{\bullet}$	$ABTS^{\bullet}$	β-carotene	FRAP	Metal chelating	ORAC
EE	48.62	13.92	29.59	15.66	64.20	7.26
MF	73.29	16.13	16.95	22.28	67.98	7.44
F1	86.01	21.75	27.51	35.73	81.52	10.33
F2	28.55	7.95	11.07	7.41	60.07	3.64
F3	22.01	5.66	12.39	5.43	53.58	4.14
F4	15.05	4.00	8.92	3.95	34.18	2.70
F5	17.33	3.38	10.28	4.66	41.96	2.95
F4.1	21.59	8.48	22.12	5.61	43.08	2.33
F4.2	140.84	23.58	*	46.29	25.33	2.14
Trolox	13.83	8.10	*	11.76	*	1.79
Quercetin	6.22	3.56	11.03	4.51	62.21	0.44
Gallic acid	*	*	*	*	2.46	*

* not calculated

flavonols exhibiting a catechol group in the B-ring (two vicinal hydroxyl groups) as having higher antioxidant activity. Antioxidant activity of quercetin derivatives has already been reported by Heim et al. (2002) who attributed the higher radical scavenging activity to quercetin aglycone, followed by *O*-methylated and *O*-glycosylated derivatives.

Furthermore, the combination of the catechol group with a double bond between C2–C3 plus a hydroxyl group in position 3 enhances the antioxidant capacity of a substance, for example, the flavonol quercetin (van Acker et al. 1996). The low antioxidant capacity of F4.2 corroborates with the proposal of van Acker et al. (1996): flavonol without a catechol group, such as derivatives of kaempferol, has lower antioxidant activity.

However, metal chelating activity assay revealed F4.2 as the fraction with the highest potential. It is not common that kaempferol derivatives present high metal chelating activity. Some studies had positively related the presence of hydroxyl groups to the metal chelating activity (Mira et al. 2002; Kumar and Pandey 2013a, b; Kumar et al. 2013), and it is known that kaempferol has one hydroxyl group lesser than quercetin. In addition, kaempferol derivative in F4.2 has a methoxyl replacing the hydroxyl group at C3, which would further reduce its activity. However, this subfraction has the presence of two quercetin derivatives closer in abundance to F5 (composed exclusively of quercetin 3-*O*-methyl ether). Presence of quercetin derivatives could explain the metal chelating activity of this sample.

Rather the other tests, measuring the oxygen radical absorbance capacity (ORAC), F4.1 showed increased activity. This fraction is composed mostly by compound 14 (quercitrin). Kim and Shim (2014), tested the hypothesis that methanol extract of *Houttuynia cordata* Thunb (MEH) and its targeted bioactive components including rutin, quercitrin, and chlorogenic acid can be effective in reducing reactive oxygen species (ROS) caused by nicotine and promoting conversion of nicotine to cotinine in HepG2 cell. Compared to nicotine only, pretreatments of MEH, rutin, and quercitrin were revealed to effectively inhibit ROS production in HepG2 cell by up to 9, 7.4, and 14 %, respectively. Boligon et al. (2012) demonstrated that quercitrin showed protective effects against damages caused by hydrogen peroxide in human lymphocytes, possibly by decreasing ROS.

Antioxidants can be synthesized in vivo or, for animals, obtained through diet. The most well-known exogenous antioxidants coming from dietary are vitamins A, C, and E and polyphenolics (Tripathi et al. 2007). Antioxidant activities of phenolic compounds, such as flavonoids, tannins, and phenylpropanoids are characterized by the redox property assigned to them, which can play an important role neutralizing reactive oxygen species (ROS) (Zheng and Wang 2001; Martin et al. 2013; Kumar and Pandey 2013a).

Medical effectiveness of many flavonoids is well established. Quercetin, for example, is the subject of many studies reporting antibacterial, hepatoprotective, anti-inflammatory, antitumor, and antiviral activities, and for all of those activities, the presence of a free hydroxyl group in C3, as well as, two vicinal hydroxyl groups in B-ring, seem to be important to enhance the flavonoid activity (van Acker et al. 1996; Kumar and Pandey 2013a).

Table 4 shows the effective concentration of each sample to achieve 50 % of the antioxidant activity (EC_{50}). For some samples, a linear correlation between concentration and antioxidant activity was not observed, and EC_{50} was not calculated. EC_{50} reveals the real antioxidant activity of a sample, once it takes into account the dose–response curve.

F4 showed the lowest EC_{50} (μg mL^{-1}) in $DPPH^{\bullet}$, β-carotene and FRAP assays, as expected. In metal chelating activity and ORAC, F4.2 exhibited the highest activity and F5 was the most active fraction in $ABTS^{\bullet}$. Comparing EC_{50} of the standards used in this study (Table 4), quercetin had higher antioxidant activity than Trolox. However, quercetin showed lower metal chelating activity compared with gallic acid. In almost tests, F4 and F5 presented EC_{50} values close to that presented by quercetin. Both fractions are mainly composed by the flavonol quercetin 3-*O*-methyl ether (substance 17).

There are many methods to assess the antioxidant capacity of a plant extract. Thus, it becomes difficult to choose the most appropriate method in order to avoid incorrect interpretation of results. A comparison of different studies is also difficult, and because of this, it is preferred to carry out a battery of assays, analyzing different mechanisms of antioxidant action and comparing them to synthetic antioxidant standards (such as Trolox, BHT, among others). According to Oliveira et al. (2009), in vitro methods must be developed carefully, taking into account the use of oxidants and targets of biological significance, such as proteins, triacylglycerol, and cellular models, as closest as possible to in vivo conditions. Furthermore, in vivo tests are necessary for confirmation of the observed activity and potential side effects of an extract or substance.

In conclusion, this research suggested *C. sphaerogynus* as promising to use in the exploration of bioactive substances, especially for antioxidant applications. Fractions showed EC_{50} values close to those presented by commercial standards as Trolox and quercetin. Further investigation and research is needed regarding the biological in vitro and particularly in vivo activities of extracts from *C. sphaerogynus*.

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