Phytomedicine 19 (2012) 953–957

Phytomedicine

journal homepage: www.elsevier.de/phymed

Short communication

Chemical composition and vasodilatation induced by *Cuphea carthagenensis* preparations

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ARTICLE INFO

Keywords:
Cuphea carthagenensis
Total phenolic contents
Quercetin5-*O*-β-glucopyranoside
Quercetin-3-*O*-α-arabinofuranoside
Vasodilator effect

ABSTRACT

The aerial parts of *Cuphea carthagenensis* (Jacq.) J.F. Macbride (Lythraceae) are traditionally employed in Brazil to treat cardiovascular diseases. The aim of this study was to compare preparations of *C. carthagenensis* aerial parts (aqueous and ethanol extracts, together with derived fractions) with regard to their total phenolic contents and *in vitro* vasodilating activity. The main flavonoids found in the extracts were isolated and identified as quercetin derivatives. The extracts and fractions showed similar HPLC profiles with the presence of quercetin-5-*O*-β-glucopyranoside, quercetin-3-*O*-α-arabinofuranoside and quercetin-3-sulfate in all of them, but marked differences in the contents of flavonoids, proanthocyanidins, tannis and total phenolics. Excepting the aqueous extract, all assayed preparations elicited vasodilatation on pre-contracted rat aortic rings in the range of pIC_{50} 4.53 ± 0.03 to 4.98 ± 0.06. Polynomial regression analysis demonstrated the relationship between vasodilating activity and the contents of flavonoids (r^2 = 0.5190), proanthocyanidins (r^2 = 0.8016), tannins (r^2 = 0.8041) and total phenolics (r^2 = 0.6226), suggesting the participation of these compounds in the pharmacological effect and their potential use as chemical markers for the species.

Introduction

Cuphea carthagenensis (Jacq.) J.F. Macbride (Lythraceae) is an herbaceous species widely spread in Brazil and other American countries, Hawaii and South Pacific islands (Graham et al. 2006). The species is popularly named *sete-sangrias* in the country, being its aerial parts traditionally used as infusion or decoction to treat circulatory disorders and heart diseases (Marodin and Baptista 2001; Lorenzi and Matos 2008), including arterial hypertension (de Lima et al. 2007; Lorenzi and Matos 2008), arteriosclerosis (Wrigley and Hayes 2000; Lorenzi and Matos 2008), pain in legs, varicose (Vendruscolo and Mentz, 2006) and to activate circulation (Vendruscolo and Mentz, 2006). In view of its extensive traditional use, *C. carthagenensis* was included in the Non-Exhaustive List of Customary Names Used in Brazil Associated to Biodiversity (Brasil 2006).

Despite the widespread use of *C. carthagenensis* in Brazil, studies on its chemical composition and pharmacological effects are still incipient. The crude extract of the aerial parts has been reported to inhibit *in vitro* angiotensin-I converting enzyme (Braga et al. 2000) and to induce vasodilatation on rat aorta preparations, probably by stimulating nitric oxide production (Schuldt et al. 2000), along with *in vitro* antioxidant activity (Schuldt et al. 2004). The butanolic fraction derived from the crude extract also induced vasodilatation and the participation of phenolic compounds in the effect has been suggested (Schuldt et al. 2000). Infusions of *C. carthagenensis* leaves were found to present hypocholesterolemic effect in rats without affecting triglyceride level and body weight (Biavatti et al. 2004). A sub-chronic study carried out for 90 days with a decoction from aerial parts suggested the absence of toxic effects, despite an increase in hepatic vascularization and hepatocyte size (Biavatti et al. 2004).

The chemistry of *C. carthagenensis* is poorly known and comprises triterpenes such as carthagenol (González et al. 1994). Recently, we reported the isolation of quercetin-3-sulfate from the aerial parts and suggested it as a chemical marker, since no sulfated flavonoid has been ever reported to a Lythraceae species (Krepsky et al. 2010). On the other hand, several flavonols, flavones and flavanones, along with their glycosides and glucuronic acid conjugates have been isolated from other *Cuphea* species (dos Santos et al. 1995; Calzada 2005). Within this context, the aim of this study

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0944-7113
http://dx.doi.org/10.1016/j.phymed.2012.05.011

was to compare preparations of *C. carthagenensis* aerial parts with regard to their total contents of flavonoids, tannins, proanthocyanidins and phenolics and *in vitro* vasodilating activity.

Materials and methods

Plant material

C. carthagenensis aerial parts were donated by Klabin Florestal do Paraná, collected in the municipality of Telêmaco Borba (collection sites at 24°19′26″S and 50°36′56″S), Brazil, in October 2005. The medicinal plants harvested on this farm are certified by a Forest Stewardship. The specimen was identified by Prof. Dr. C.M. Sakuragui (Universidade Estadual de Maringá – UEM, Brazil) and a voucher specimen (147 HKLABIN) is deposited at the Herbarium of UEM.

Preparation of extracts and fractions

The aerial parts were dried in a ventilated oven at 40 °C, for 72 h. The dried material was ground in a knife mill (1000 g) and extracted by exhaustive percolation with ethanol. The extract was concentrated to residue by removing the solvents in a rotavapor, at 50 °C, furnishing a dark green residue (171 g). Portions of it (30 g) were suspended in water (300 ml) and sequentially partitioned with equal volumes (3 × 100 ml) of *n*-hexane, DCM, EtOAc and *n*-BuOH. Solvents were removed in a rotavapor, at maximum temperature of 50 °C. The process was repeated 5 times to afford the *n*-hexane (34.7 g), DCM (2.6 g), EtOAc (3.0 g), *n*-BuOH (13.9 g) and water (84.2 g) fractions, along with an emulsion (1.7 g) generated during the partition of the extract with dichloromethane. MeOH (50 ml) was added to the water fraction residue to afford the soluble (50.6 g) and insoluble (27.5 g) parts. The aerial parts (18 g) were also extracted by water (2%, w/v), at temperature of 70–80 °C, under stirring for 10 min. The aqueous extract was lyophilized affording a yellowish residue (3.3 g).

Isolation and identification of flavonoids

The compounds corresponding to the major peaks found in the HPLC chromatograms of the ethanol and aqueous extracts of *C. carthagenensis* aerial parts, recorded at 350 nm (see "HPLC profiles"), were isolated and identified. Quercetin-3-sulfate (**1**) was obtained as previously described by us (Krepsky et al. 2010). A portion of the *n*-BuOH fraction (30 mg) was subjected to preparative HPLC (Shimadzu system composed of binary pump model LC-8A and UV–Vis detector model SPD-6AV, Japan) on an ODS column (250 × 9.4 mm i.d., 5 μm, Agilent Zorbax SB, USA), sequentially eluted with methanol/water, 3.5:6.5 (0–14 min) and methanol/water, 4:6 (14.1–25 min) at a flow rate of 3.0 ml/min, at room temperature, to afford compound **2** (RT = 20 min) as an amorphous yellowish solid (8.0 mg). The EtOAc fraction (100 mg) was chromatographed over an ODS column (250 × 21.1 mm i.d., 10 μm, Agilent Prep, USA) eluted with methanol/water acidified with 1% HAc (3:7) at a flow rate of 8.0 ml/min. The process was carried out at room temperature, using the Shimadzu system described above to give compound **3** (TR = 40.9 min) as an amorphous yellowish solid (9.9 mg).

Structure elucidation of compounds **2** and **3** was accomplished by spectroscopic analysis, including UV data obtained with shift reagents (Mabry et al. 1970) and by 1D and 2D NMR experiments (DEPT-135, HSQC and HMBC), and also by comparison with literature data (Tamura et al. 2002; Ek et al. 2006). NMR spectra were recorded on a Bruker Avance DPX400 equipment (Germany) operating at 100 MHz for ^{13}C and 400 MHz for ^{1}H. DMSO-*d6* was employed as solvent and TMS was used as internal reference for both nuclei. Based on the obtained spectral data and by comparison with literature records, compounds **2** and **3** were respectively identified as quercetin-5-*O*-β-glucopyranoside and quercetin-3-*O*-α-arabinofuranoside (Fig. 1).

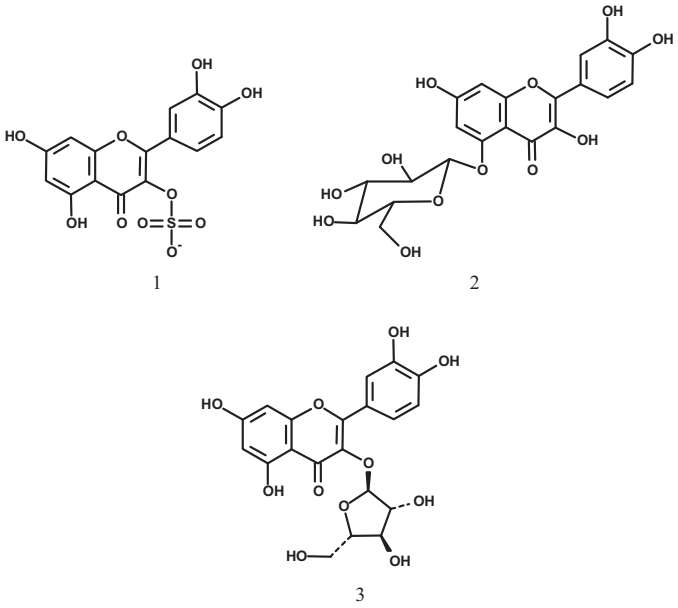

Fig. 1. Chemical structures of the flavonoids identified in preparations from *C. carthagenensis* aerial parts. (1) Quercetin-3-sulfate; (2) quercetin-5-*O*-β-glucopyranoside; (3) quercetin-3-*O*-α-arabinofuranoside.

HPLC profiles

Analyses were carried out on a Waters alliance 2695 HPLC system composed of a quaternary pump, an auto sampler, a photodiode array detector (DAD) 2996 and a Waters Empower pro data handling system (Waters Corporation, USA). An ODS column (125 × 4.0 mm i.d., 5 μm; Merck, Germany) in combination with a LiChrospher 100 RP-18 guard column (4 mm × 4 mm i.d., 5 μm; Merck, Germany) was employed for the analysis. A segmented gradient was used for elution, at 40 °C and flow rate of 1.0 ml/min, as follows: 0 min 95% A, 5% B; 2 min 88% A, 12% B; 18 min 80% A, 20% B; 20 min 72% A, 28% B. The detection was set at 350 nm and UV spectra data from 190 to 400 nm were recorded on line. For the analyses, the samples were dissolved in methanol (Tedia, USA) to concentrations of 1 mg/ml. After centrifugation at 10,000 rpm, for 10 min, the sample solutions (10 μl) were automatically injected onto the HPLC system.

Spectrophotometric quantification

Analyses were carried out on an UV/Vis spectrophotometer Lambda 20 (Perkin Elmer, Waltham, MA, USA). The total flavonoid contents were assayed in the extracts and fractions by measuring at 425 nm the absorbance of the complex formed with aluminum chloride in acidic medium, according to the method described for Calendulae analysis in the European Pharmacopoeia (Eur. Pharm. 2004). A five-point calibration curve was constructed by plotting the absorbance data and concentrations of standard quercetin solutions (1–12 μg/ml). The total tannin concentration was assayed by measuring at 715 nm the absorbance of the product formed with Folin-Denis reagent, according to the method described for *Stryphnodendron adstringens* in the Brazilian Pharmacopoeia (Farm. Bras 2002). PVPP was employed for tannin adsorption in substitution to skin powder and the method has been validated accordingly (data

not shown). A five-point calibration curve was obtained by plotting the absorbance data and concentrations of standard pirogalol solutions (0.313–3.75 μg/ml). Total phenolics were quantified by the same method, measuring the absorbance of the product with Folin-Denis reagent before the absorption of tannins by PVPP. For the quantitation of flavonoids and tannins, curves were obtained in three different days and had their statistical equivalency evaluated by ANOVA. The curves showed satisfactory linearity within the analyzed range ($r^2 > 0.99$). Total proanthocyanidin content was determined by measuring at 540 nm the absorbance of the resulting cyanidin chloride, after acid-catalyzed solvolysis with *n*-BuOH/HCl 37% (95:5), according to the method described for *Crataegus* in the European Pharmacopoeia (Eur. Pharm. 2005). Analyses were performed in triplicates and the results are presented as mean ± standard deviation (% w/w, ±s.d.).

Vasorelaxant activity

Male Wistar rats (200–250 g) from the Animal Care Facilities of Instituto de Ciências Biológicas, UFMG, were used. They were kept at 22–25 °C in a 12 h light/dark cycle, and had free access to food and water. Animal experiments were performed according to the recommendations of the Brazilian Council for Animal Care. The project was approved by the research ethics committee at UFMG (protocol number 17/2008). The descending thoracic aorta were prepared and mounted as previously described (Cortes et al. 2002). The determination of vasorelaxant activity was performed in aortic rings with functional endothelium, pre-contracted with phenylephrine (0.1 μmol/l). The extracts and fractions were added in increasing cumulative concentrations, once the response to phenylephrine had stabilized.

Statistical analysis

The results are expressed as mean ± standard deviation of at least five experiments. Values of −log of the concentration (g/ml) required to produce 50% of relaxation of sustained contraction induced by phenylephrine (pIC_{50}) were calculated graphically from the individual concentration–response curves by non-linear curve fitting. The statistical analyses were performed with one-way ANOVA plus Tukey post-test for the pIC_{50} ($p < 0.05$). The relationship between the contents of phenolics (tannins, flavonoids, proanthocyanidins and total phenolics, expressed as percentage of dry material, w/w) and the vasodilator effect (pIC_{50}) was analyzed by second order polynomial regression, using Prisma 5.01 software (GraphPad, USA).

Results and discussion

A previous study suggested phenolic compounds found in the *n*-BuOH fraction as responsible for the vasodilating activity of *C. carthagenensis* (Schuldt et al. 2000). Therefore, in order to compare the chemical composition and biological activity of *C. carthagenensis* preparations, in the present work they were characterized by their HPLC profiles and contents of phenolics (flavonoids, tannins, proanthocyanidins and total phenolics). Since the chemistry of the species is poorly known, we undertake the phytochemical study of the flavonoid-rich fractions of *C. carthagenensis* which resulted in the isolation of quercetin-5-*O*-β-glucopyranoside (**2**) and quercetin-3-*O*-α-arabinofuranoside (**3**), respectively from the *n*-BuOH and EtOAc fractions (Fig. 1). Both compounds are here described for the first time for a *Cuphea* species.

The HPLC fingerprints registered at 350 nm for the ethanol and water extracts indicated the presence of four major peaks, which were identified by comparison with the retention times and co-injection of isolated compounds as quercetin-3-sulfate (**1**, RT = 9.1 min), quercetin-5-*O*-β-glucopyranoside (**2**, RT = 11.0 min) and quercetin-3-*O*-α-arabinofuranoside (**3**, RT = 15.4 min), along with a non-identified quercetin glucoside (**4**, λ_{max} 257 and 354 nm) (Fig. 2). Quercetin-3-sulfate was identified as the major peak of the emulsion, ethanol and water extracts (Fig. 2). The ethanol and water extracts presented similar qualitative composition (Fig. 2), with higher concentrations of flavonoids, tannins, proanthocyanidins and total phenolics in the first (Table 1). The aqueous fraction was divided into methanol soluble and insoluble parts, and both showed qualitative composition similar to the water extract (chromatograms not shown). The MeOH-insoluble water fraction presented the lowest concentration of all assayed compounds, excepting the total phenolic content which was lower in the water extract (Table 1). On its turn, the *n*-BuOH fraction exhibited the highest content of tannins (75.0 ± 0.9%) and total

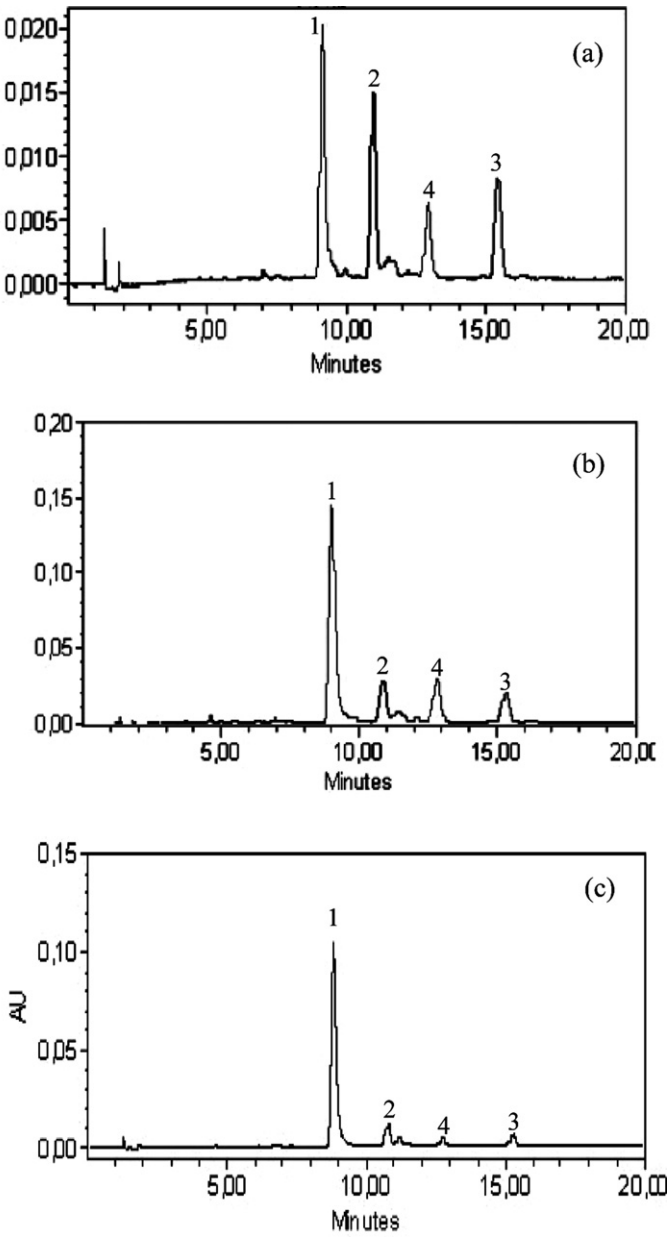

Fig. 2. HPLC profiles of preparations from *C. carthagenensis* aerial parts. (a) Ethanol extract; (b) water extract; (c) emulsion. Peaks: (1) quercetin-3-sulfate; (2) quercetin-5-*O*-β-glucopyranoside; (3) quercetin-3-*O*-α-arabinofuranoside; (4) non-identified quercetin glucoside. Chromatographic conditions: see experimental part.

Table 1
Vasodilator effect and quantitative composition of preparations from *C. carthagenensis* aerial parts.

Preparation	Maximum vasodilatation (% ±s.d.)*	Vasodilatation (pIC_{50} ± s.d.)*	Total contents (% ±s.d., $n = 3$)			
			Flavonoids	Tannins	Proanthocyanidins	Phenolics
EtOH extract	81.8 ± 5.1[b]	4.92 ± 0.11[a]	1.86 ± 0.05	38.3 ± 3.4	14.60 ± 0.50	45.8 ± 1.1
Water extract	46.8 ± 14.4[c]	nc[a]	1.04 ± 0.04	11.8 ± 0.4	4.78 ± 0.22	17.8 ± 0.4
n-BuOH fraction	86.2 ± 1.6[a,b]	4.98 ± 0.06[a]	4.27 ± 0.04	75.0 ± 0.9	19.80 ± 1.50	87.6 ± 4.2
MeOH-insoluble water fraction	94.8 ± 4.3[a]	4.53 ± 0.03[b]	0.37 ± 0.01	11.2 ± 0.1	3.46 ± 0.30	18.7 ± 0.4
MeOH-soluble water fraction	89.1 ± 4.5[a,b]	4.85 ± 0.11[a]	0.42 ± 0.01	38.6 ± 1.0	9.61 ± 0.09	46.3 ± 1.2
Emulsion	86.0 ± 7.1[a,b]	4.93 ± 0.07[a]	5.80 ± 0.16	36.5 ± 1.5	37.90 ± 0.50	61.3 ± 4.1

[a] Not calculated (maximum vasodilatation not enough to calculate pIC_{50}).
[*] Distinct letters within the columns indicate significant differences among the values ($p < 0.05$).

phenolics (87.6 ± 4.2), while proanthocyanidins (37.90 ± 0.50%) concentrated in the emulsion produced during partition of the ethanol extract with dichloromethane. Besides, the total flavonoid content (5.80 ± 0.16%) reached the highest concentration in the emulsion, quercetin-3-sulfate identified as its major peak (Fig. 2). The preparations presented different chemical constitutions and their vasodilator effects were assayed to investigate quantitative composition–activity relationships.

The maximal dilatation (46.8 ± 14.4%) elicited by the aqueous extract was below 50%, which was significantly lower than the response induced by all other preparations (81.8 ± 5.1% to 94.8 ± 4.3%); therefore, its pIC_{50} value was not determined. All other extracts and fractions elicited a concentration-dependent relaxant effect in vessels containing functional endothelium with pIC_{50} in the range from 4.53 ± 0.03 to 4.98 ± 0.06 (Table 1). The vasodilatation induced by the ethanol extract, emulsion, *n*-BuOH fraction and MeOH-soluble water fraction were statistically equivalent ($p > 0.05$) and differ from the response elicited by the MeOH-insoluble water fraction. Taken together, the pharmacological data suggest that there are no differences between the vasodilator effect of the ethanol extract and derived fractions. Therefore, extract fractionation seems to offer no advantage in pharmacological terms. This hypothesis is sustained by the absence of vasorelaxant activity observed for quercetin-3-sulfate (Krepsky et al. 2010), although the emulsion, enriched in this compound, elicited a strong response. It should be remembered, however, that quercetin-3-sulphate undergoes *in vivo* transformation into quercetin, whose vasodilatation effect is well characterized (Ajay et al. 2003).

In order to investigate the potential participation of the assayed metabolites in the biological activity, the contents (% w/w) of flavonoids, proanthocyanidins, tannins and total phenolics were submitted to non-linear regression analysis with the vasodilator effect (pIC_{50} values) elicited by the preparations. Second order polynomial equations were estimated to describe the relationship between vasodilator effect and concentrations of flavonoids ($r^2 = 0.5190$ $y = 4.618 + 0.1993x - 0.0254x^2$), proanthocyanidins ($r^2 = 0.8016$ $y = 4.400 + 0.0497x - 0.0002x^2$), tannins ($r^2 = 0.8041$ $y = 4.300 + 0.0228x - 0.0002x^2$) and total phenolics ($r^2 = 0.6226$ $y = 4.210 + 0.0206x - 0.0001x^2$), pointing out the participation of these metabolites in the biological effect induced by the preparations. Graphic representation of the calculated curves allows identifying intervals of concentrations associated with the maximum biological response (Fig. 3).

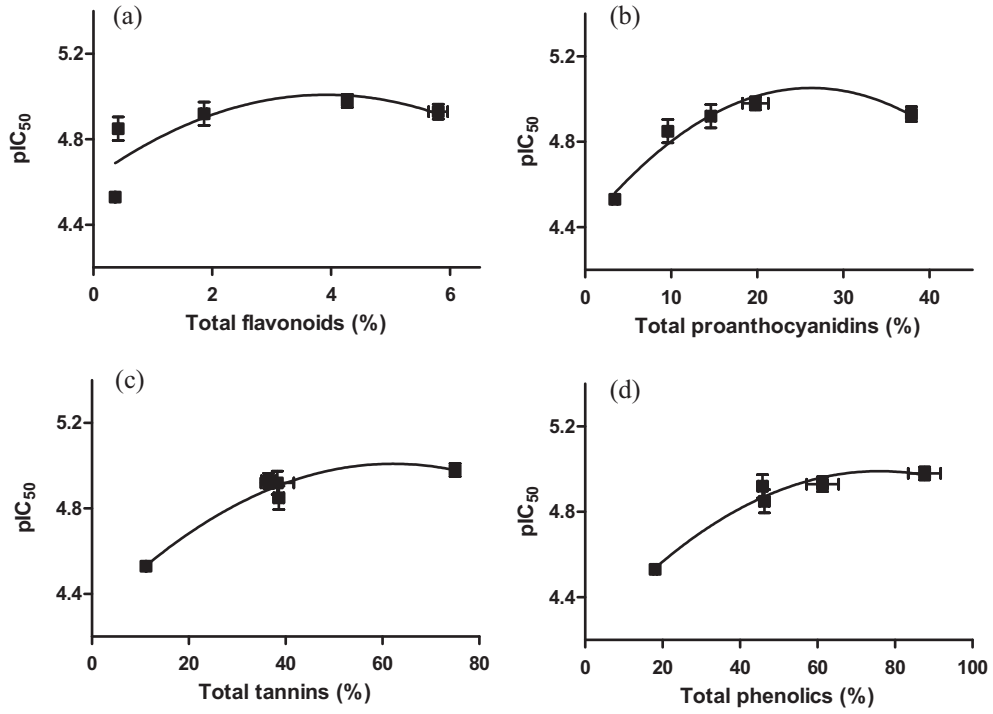

Fig. 3. Polynomial regression (quadratic) curves between the concentrations of phenolics and the vasodilator effect (pIC_{50}) induced by preparations of *C. carthagenencis*. (a) Flavonoids; (b) proanthocyanidins; (c) tannins; (d) total phenolics.

In the same direction, strong correlations between the contents of phenolics and antioxidant/vasorelaxant activities of medicinal plants have been recently reported (Ibarra-Alvarado et al. 2010; Chew et al. 2011), corroborating the potential benefit of these compounds in cardiovascular diseases. This assumption is also supported by results obtained for red wines, associating the vasodilatory effect with the concentration of total phenolics (Mudnic et al. 2011) or of specific flavonoids like kaempferol (Padilla et al. 2005). Therefore, it is feasible to suppose that the vasodilatation elicited by *C. carthagenensis* preparations is related to their phenolic constituents, whose anti-oxidant properties have been previously demonstrated (Schuldt et al. 2004).

Proanthocyanidins are regarded as the bioactive constituents of *Crataegus* species, employed in the treatment of hypertension. The standardized *Crataegus* extract WS 1442, currently in clinical use, induced vasodilatation in rat vessel rings pre-contracted by phenylephrine (IC_{50} 15.1 ± 0.6 μg/ml; Brixius et al. 2006), with magnitude similar to the preparations of *C. carthagenensis* here described, demonstrating the potential therapeutic use of the species.

In conclusion, our findings support the participation of flavonoids, proanthocyanidins and tannins in the vasorelaxant effect of *C. carthagenensis* and point out synergistic effects between these constituents in the ethanol extract. Such compounds might be used as chemical markers for the quality control of the vegetal drug and derived preparations of *C. carthagenensis* aerial parts.

Acknowledgments

We acknowledge FAPEMIG/Brazil and CNPq/Brazil for the financial support and research fellowships (F.C.B., S.F.C.). CAPES/Brazil is acknowledged for a PhD fellowship (P.B.K.). We are also thankful to Loana Aparecida Pereira da Silva Johansson, Klabin do Paraná Produtos Florestais Ltda, Telêmaco Borba, Paraná, Brazil, for furnishing the plant material.

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