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## Antioxidant activities and HPLC/DAD Analysis of Phenolics and Carotenoids from the Barks of *Cariniana domestica* (Mart.) Miers

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### ABSTRACT

The species *Cariniana domestica* belongs to Lecythidaceae and is popularly known as Jequitibá. The aim of the study was to evaluate the antioxidant activities from different bark extracts using DPPH and TBARS assays, as well as identify and quantify phenolic compounds and carotenoids by means of HPLC/DAD. The plasma lipid peroxidation was induced with iron sulfate in male Wistar rats and the radical scavenging activity was determined using 2,2-diphenyl-1-picrylhydrazyl (DPPH). Folin-Ciocalteu was used to determine the phenolic content. TBARS assay varied from 11.11±0.85 to 44.5±2.31 and DPPH from 6.5±0.81 to 19.5±1.32 µg mL<sup>-1</sup>. The phenolic content ranged from 54.6±0.33 to 309.3±2.73. Beta-carotene (3.73±1.02%) was found in dichloromethane fraction. Several phenolic compounds were identified in ethyl acetate fraction, being caffeic acid the most abundant compound (5.81±0.64%). Results obtained indicated that *C. domestica* exhibits great antioxidant properties.

**Key words:** *Cariniana*, polyphenols, carotenoids, TBARS, HPLC

### INTRODUCTION

In recent years, the interest in finding natural antioxidants from plant materials has been increased, as seen that crude extracts and pure natural compounds from plants were reported to have antioxidant activities (Barla *et al.*, 2007).

Oxidative stress can be defined as the imbalance between the production of Reactive Oxygen Species (ROS) and the ability of enzymatic and non-enzymatic biological systems to protect against their injury. Different therapeutic strategies have been proposed for the prevention and treatment of ROS-mediated diseases, with special emphasis on antioxidant therapy (Paz *et al.*, 2010).

Researches suggest that a wide variety of phytochemicals, such as phenolics and carotenoids may prevent oxidative stress-induced damage leading to cancer (Alesiani *et al.*, 2010). Reports have confirmed that lower incidence of age-related chronic diseases is associated to the consumption of carotene-rich vegetables (Sangheeta *et al.*, 2009). Lycopene exerts anti-inflammatory effects through its action as free radical scavenger which may reduce cellular damage (Pennathur *et al.*, 2010). β-carotene, besides it is a known quencher of ROS, is also applied in photoprotective compounds (Eicker *et al.*, 2003).

*Cariniana domestica* belongs to Lecythidaceae and is known in Brazil as jequitibá-roxo. Lecythidaceae is composed of 10 genera and 230 species, being a dominant group in neotropical forests (Tsou and Mori, 2007). The bark infusion of these species is commonly used as antioxidant, anti-inflammatory and antimicrobial remedies (Neto and Morais, 2003; Silva *et al.*, 2009). Preliminary phytochemical investigations of our group concerning the barks of *C. domestica* demonstrated the presence of triterpenoids and phenolic compounds (Janovik *et al.*, 2011). This feature allied to the importance of the oxidative stress in the pathogenesis of various diseases led us to better evaluate the antioxidant potential of this plant. Simultaneously, phytochemical analysis led identification of several phenolic compounds, besides lycopene and  $\beta$ -carotene and its HPLC/DAD analysis.

## MATERIALS AND METHODS

**Chemicals, apparatus and general procedures:** Folin-Ciocalteu and solvents for the extractions, dichloromethane, ethyl acetate, ethanol, methanol and n-butanol were purchased from Merck (Darmstadt, Germany). Ascorbic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH), quercetin, rutin, kaempferol, gallic, chlorogenic and caffeic acids standards were acquired from Sigma Chemical Co. (St. Louis, MO, USA).  $\beta$ -carotene (>95%) and lycopene were acquired from Sigma-Aldrich. High performance liquid chromatography (HPLC-DAD) was performed with the HPLC system (Shimadzu, Kyoto, Japan), Prominence Auto-Sampler (SIL-20A), equipped with Shimadzu LC-20AT reciprocating pumps connected to the degasser DGU-20A5 with integrator CBM-20A, UV-VIS detector DAD SPD-M20A and software LC Solution 1.22 SP1. Absorbances were measured in a Shimadzu-UV-1201.

**Plant collection and extractions:** The barks of *C. domestica* were collected in Tangará da Serra-Mato Grosso do Sul, Brazil, in March of 2008. Exsiccate was archived as voucher specimen in the herbarium of Department of Pharmacology at UFSM (SMDB 11818).

Plant material was dried at room temperature and powdered. The powder was macerated with ethanol 70% (v/v) during ten days. After filtration, the extract was evaporated under reduced pressure. This procedure was repeated twice and the extracts were combined. The remained aqueous extract obtained was partitioned with solvents in increasing polarity: dichloromethane, ethyl acetate and n-butanol. At the end of all extractions procedures, each extract was evaporated under reduced pressure to obtain the dried fractions.

**Radical-scavenging activity: DPPH assay:** Antioxidant activity was evaluated by monitoring its ability in quenching the stable free radical DPPH, according to a slightly modified method previously described (Kussar *et al.*, 2006). The DPPH quenching ability was expressed as IC<sub>50</sub>. Six different ethanol dilutions of each fraction and crude extracts at 250, 125, 62.5, 31.25, 15.62 and 7.81  $\mu\text{g mL}^{-1}$  were mixed with 1.0 mL of DPPH 0.3 mM in ethanol solution. After 30 min, the readings were made at 518 nm. A solution of DPPH (1 mL, 0.3 mM) in ethanol (2.5 mL) was used as a negative control and ascorbic acid in the same concentrations used for the fractions and the crude extracts provide the positive control. The test was performed in triplicate and the calculation of the antioxidant activity followed the equation:

$$\text{Inhibition (\%)} = 100 - \frac{(\text{Abs}_{\text{sample}} - \text{Abs}_{\text{blank}})}{\text{Abs}_{\text{control}}} \times 100$$

where,  $Abs_{sample}$  is absorbance of each fraction,  $Abs_{blank}$  is absorbance of fractions without adding the DPPH and  $Abs_{control}$  is absorbance the solution of ethanol in DPPH.

The percentage of inhibition was calculated and a graphic of percentage of inhibition versus concentration was constructed. Correlation coefficients were optimised (Tsimogiannis and Oreopoulou, 2006).

**Determination of total phenolics:** The determination of total phenolic content was performed by the Folin-Ciocalteu method (Chandra and Mejia, 2004). Briefly, 0.5 mL of 2 N Folin-Ciocalteu reagent was added to a 1 mL of each sample ( $0.15 \text{ mg mL}^{-1}$ ) and this mixture was allowed to stand for 5 min before the addition of 2 mL of 20%  $\text{Na}_2\text{CO}_3$ . The solution was then allowed to stand for 10 min before reading at 730 nm. Total phenolic content was expressed in milligrams equivalents of Pyrogalllic acid (PGAE) per gram of each fraction. The equation obtained for the calibration curve of pyrogalllic acid in the range of 0.005 to  $0.030 \text{ mg mL}^{-1}$  was  $y = 34.443x - 0.0942$  ( $r^2 = 0.9984$ ).

**In vitro Fe(II)-induced lipid peroxidation in rats' brain:** Male Wistar rats weighing 270-320 g and with age from 3 to 3.5 months, from our own breeding colony were kept in cages of 3 or 4 animals each, with continuous access to food and water in a room with controlled temperature ( $22 \pm 3^\circ\text{C}$ ) and on a 12 h light/dark cycle with lights on at 7:00 a.m. The animals were maintained and in accordance to the guidelines of the Brazilian Association for Laboratory Animal Science (COBEA).

Rats were decapitated under mild ether anesthesia and the encephalic tissue was rapidly dissected and placed on ice. Tissues were immediately homogenized in cold 10 mM Tris-HCl, pH 7.5 (1/10, w/v). The homogenate was centrifuged for 10 min at 4000 xg to yield a pellet that was discarded and a low-speed Supernatant (S1) was used for the TBARS assay (Puntel *et al.*, 2005).

After centrifugation, an aliquot of 100  $\mu\text{L}$  of S1 was incubated for 1 h at  $37^\circ\text{C}$  with pro-oxidants agent (10  $\mu\text{M}$  of Iron Sulfate) in presence or absence of plant extracts and then used for TBARS determination. TBARS production was determined as described by Puntel *et al.* (2005). Data were analyzed statistically by one-way ANOVA, followed by Duncan's multiple range tests.

**HPLC-DAD qualitative and quantitative analysis of polyphenols:** Reverse phase chromatography analysis were carried out with a Phenomenex  $\text{C}_{18}$  column ( $4.6 \times 250 \text{ mm}$ ) packed with 5  $\mu\text{m}$  diameter particles, volume injection was 40  $\mu\text{L}$  and the gradient elution was conducted according to the Evaristo and Leitao (2001) method. The UV absorption spectra of the standards as well as the samples were recorded in the range of 230-400 nm.

Stock solutions of standards were prepared in methanol in the range of  $0.0025$ - $0.045 \text{ mg mL}^{-1}$ . Quantification was carried out by the integration of the peaks using external standard method, considering the 254 nm for gallic acid, 327 nm for chlorogenic and caffeic acids and 365 nm for the three flavonoids (quercetin, rutin and kaempferol). All the samples and standards solutions as well as the mobile phase were degassed and filtered through 0.45  $\mu\text{m}$  membrane filter (Millipore). The chromatographic operations were carried out at ambient temperature and in triplicate.

**HPLC-DAD analysis of carotenoids:** Standard solutions of  $\beta$ -carotene were prepared in mobile phase, at concentrations from 0.2 to  $0.6 \text{ mg mL}^{-1}$ . The dichloromethane fraction was dissolved in mobile phase. Each concentration was injected three times and the calibration curve was obtained by linear regression.

The  $\beta$ -carotene analysis was carried out under isocratic conditions using a  $C_8$  column (4.6×250 mm), packed with 5  $\mu$ m diameter particles. The mobile phase was acetonitrile:methanol:ethyl acetate with 1% of acetic acid (88:8:4, v/v/v) and the flow rate, 0.8 mL min<sup>-1</sup>. Detections were done at 453 nm and the injection volume was 60  $\mu$ L. Identification of  $\beta$ -carotene was achieved by comparing retention time and UV/DAD spectra with those of standard. All chromatographic operations were performed at room temperature and in triplicate.

**Statistical analysis:** Data from the TBARS assay were analyzed statistically by one-way Analysis of Variance (ANOVA), followed by Duncan's multiple range tests when appropriated using the statistical software SPSS 10.0 for Windows. TBARS graphics were constructed using the Slide Write 4.032 Bit Edition program. Statistical p-values were calculated to quantify levels of significance for each treatment type. A significant p-value ( $p < 0.05$ ,  $p < 0.01$ , or  $p < 0.001$  when appropriate) means that there exists significant difference between the two sets of data being analyzed. Correlation coefficient (r) to determine the relationship between two variables and the standard deviations in the DPPH and total phenolics assays were calculated from the data obtained from three separate experiments using MS Excel for Windows.

## RESULTS AND DISCUSSION

Total Phenolic contents (TP) assayed by the method of Folin-Ciocalteu expressed in milligrams equivalent of pyrogallol acid (PGAE) per gram of each fraction and  $IC_{50}$  determinations by the DPPH method are given in Table 1.

The quantitative determination of the polyphenolic content of *C. domestica* used Folin-Ciocalteu as the main reagent and pyrogallol acid as reference. In order, the phenolic contents obtained were ethyl acetate fraction > crude extract > butanolic fraction > dichloromethane fraction. Investigations of qualitative composition of plant extracts revealed the presence of high concentrations of phenolics in the extracts obtained using polar solvents (Canadanovic-Brunet *et al.*, 2008).

Comparing the content of polyphenolic and antioxidant activity ( $IC_{50}$ ), we can correlate the phenolic content of ethyl acetate fraction and its lower  $IC_{50}$ . This relationship of phenolic content and polarity is well documented (Chandra and Mejia, 2004).

Butanolic fraction exhibited larger phenolic content than dichloromethane fraction but its  $IC_{50}$  was higher. The structure of the phenolic groups and possible changes in hydroxyl, by glycosylation as an example, cause a decrease in antioxidant activity, due to the reduction in the number of hydroxyls and the steric hindrance that sugar gives, difficulting the connection to the free radical (Tsimogiannis and Oreopoulou, 2006). Dichloromethane fraction expressed good  $IC_{50}$  value (16.4±0.35  $\mu$ g mL<sup>-1</sup>) and the lowest phenolic content.

Table 1: Total phenolic content and antioxidant activity ( $IC_{50}$ /DPPH) for crude extract and fractions of *C. domestica*

Fraction	TP±SE (mg g <sup>-1</sup> )	<sup>a</sup> IC <sub>50</sub> ±SE ( $\mu$ g mL <sup>-1</sup> )	<sup>b</sup> IC <sub>50</sub> ±SE ( $\mu$ g mL <sup>-1</sup> )
Crude extract	206±3.26	12.3±1.15	18.1±2.02
Dichloromethane	54.6±0.33	16.4±0.35	44.5±2.31
Ethyl acetate	309.3±2.73	6.5±0.81	11.11±0.85
Butanol	260.0±1.96	19.5±1.32	15.2±1.14
Ascorbic acid	-	9.0±0.79	-

TP: Total phenolics expressed as pyrogallol acid equivalents (mg g<sup>-1</sup> fraction±SE), SE: Standard error, <sup>a</sup>IC<sub>50</sub>: Values for inhibition by fractions and crude extract of DPPH oxidation capacity, <sup>b</sup>IC<sub>50</sub>: Values for inhibition by fractions and crude extract of TBARS production induced by Fe(II) in brain preparations

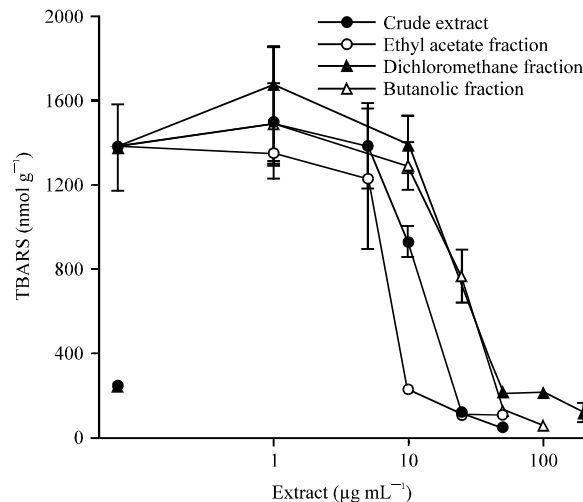


Fig. 1: Effects of different concentrations of crude extract, ethyl acetate, dichloromethane and butanolic fractions from the barks of *C. domestica* on Fe(II) (10 µM)-induced TBARS production in brain homogenates. The samples were incubated for 1 h or without (basal) with Fe(II) and the plant extracts. Data show means±SEM values average from 3 to 6 independent experiments performed in duplicate

In TBARS assay, free Fe (II) can induce neurotoxicity via stimulation of the Fenton reaction and its levels are increased in some degenerative disease (Bostanci and Bagirici, 2008). In this study, ethyl acetate fraction presented the best value of  $IC_{50}$  ( $11.11 \pm 0.85 \mu\text{g mL}^{-1}$ ), followed by butanol fraction ( $15.2 \pm 1.14 \mu\text{g mL}^{-1}$ ), crude extract ( $18.1 \pm 2.02 \mu\text{g mL}^{-1}$ ) (Fig. 1). Dichloromethane expressed the lowest value ( $44.5 \pm 2.31 \mu\text{g mL}^{-1}$ ). The results have shown a positive correlation between HPLC/DAD analysis, in which all tested phenolic compounds were identified and quantified in ethyl acetate fraction. Previous study developed by Pereira *et al.* (2009) evaluated the activity of flavonoids. Rutin ( $IC_{50} = 28.8 \mu\text{g mL}^{-1}$ ), quercetin ( $12.2 \mu\text{g mL}^{-1}$ ) and quercetin ( $1.4 \mu\text{g mL}^{-1}$ ) have shown good results. We can assume that phenolic compounds are closely related to inhibition of lipid peroxidation.

Within only a few years, there has been exponential growth in research to localize carotenoid in plants and numerous chromatographic methods to assay carotenoids in plants have been published (Serino *et al.*, 2009). In this study, both compounds were separated using mixture of acetonitrile: methanol: ethyl acetate with 1% of acetic acid (88:8:4, v/v/v). For quantification, a calibration curve was constructed with reference standard  $\beta$ -carotene ( $Y = 44368x - 3707.2$  with  $r = 0.9995$ ).  $\beta$ -carotene (Peak 2,  $RT = 12.5$  min) was found to represent  $3.73 \pm 1.02\%$  from whole fraction. Peak 1 was identified as lycopene.

The bark extracts *C. domestica* were investigated for the presence of the following polyphenolic compounds: gallic, chlorogenic and caffeic acids, quercetin, rutin and kaempferol. Identification of the compounds was done by comparison of their retention's time and UV/DAD absorption spectrum with those of the standards. Results obtained for this HPLC screening (Fig. 2, Table 2) indicates that ethyl acetate fraction englobes the larger number of phenolic compounds. All searched standards were found to be represented in this fraction constitution. Caffeic acid was found in major quantities, followed by chlorogenic acid and kaempferol.

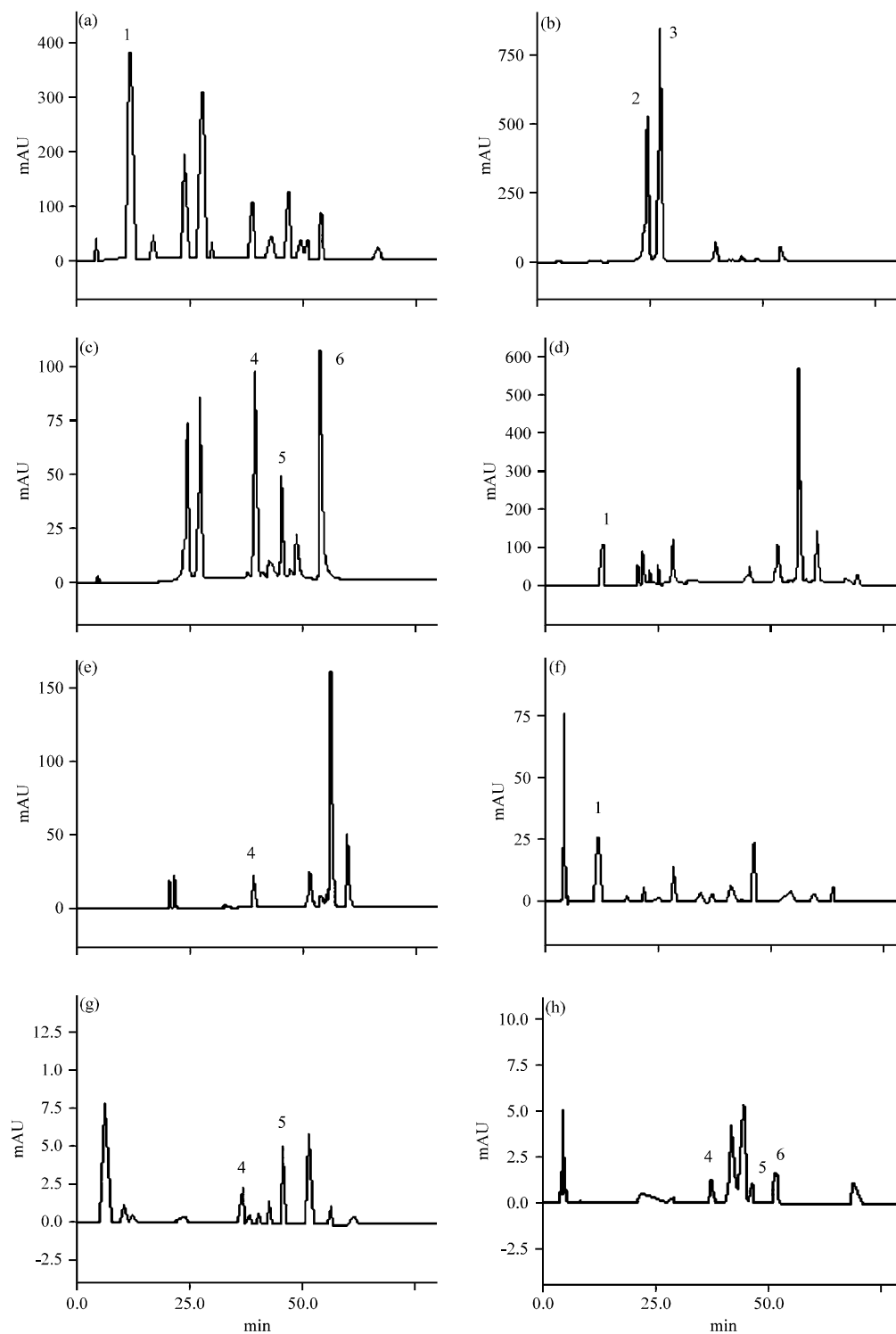


Fig. 2(a-h): HPLC/DAD chromatograms obtained for polyphenols screening. (a) Ethyl acetate at 254 nm, (b) Ethyl acetate at 327 nm, (c) Ethyl acetate at 365 nm, (d) Dichloromethane at 254 nm, (e) Dichloromethane at 365 nm, (f) Crude extract at 254 nm, (g) Crude extract at 365 nm and (h) n-butanol at 365 nm, Peaks; 1: Gallic acid, 2: Caffeic acid, 3: Chlorogenic acid, 4: Rutin, 5: Quercetin, 6: Kaempferol

Table 2: HPLC/DAD of identified and quantified polyphenols in crude extract and fractions of *C. domestica*

Fraction	Polyphenols (mg g <sup>-1</sup> of dried extract or fraction)					
	GA	CFA	CLA	RT	QT	KF
Crude extract	3.28±0.12	-	-	0.10±0.31	0.71±0.26	-
Dichloromethane	2.46±0.23	-	-	0.62±0.22	-	-
Ethyl acetate	2.83±1.12	5.81±0.64	5.38±0.12	0.18±0.25	0.96±0.83	4.16±0.54
Butanol	-	-	-	0.02±0.41	0.06±0.22	0.34±0.92

Values are Mean±standard deviation (n = 3), GA: Gallic acid, CFA: Caffeic acid, CLA: Chlorogenic acid, RT: Rutin, QT: Quercetin, KF: Kaempferol

## CONCLUSION

In this study, we could establish a positive correlation between phenolic compounds and antioxidant activity with ethyl acetate fraction which shed a light on this positive relation, as seen that it presented the highest phenolic content and the lowest IC<sub>50</sub>-DPPH and TBARS assays. HPLC/DAD analysis performed with *C. domestica* revealed the presence of important bioactive phenolic acids, flavonoids and carotenoids.

The results furnished support to assume that *C. domestica* achieves antioxidant properties, which may be related to its popular uses. Considering the absence of scientific literature related to this species, this study is a contribution to the knowledge concerning the genus *Cariniana*.

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