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HPLC/DAD Analysis, Determination of Total Phenolic and Flavonoid Contents and Antioxidant Activity from the Leaves of *Cariniana domestica* (Mart) Miers

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ABSTRACT

The species *Cariniana domestica*, popularly known in Brazil as jequitibá-roxo, belongs to Lecythidaceae and is very little studied, although it is used in folk Brazilian medicine. In this work, antioxidant potential, total phenolic and flavonoid contents from the leaves of the plant were investigated. Additionally, HPLC/DAD analysis of ethyl acetate fraction revealed five active phenolic compounds (gallic, chlorogenic and caffeic acids, rutin and kaempferol). Antioxidant potential was determined using DPPH assay. Total phenolics were measured using Folin Ciocalteau and total flavonoid, using aluminium chloride. HPLC/DAD analysis was performed using gradient system. The leaves of *C. domestica* exhibited high antioxidant activity, as well as a large content of phenolic compounds and flavonoids. IC_{50} obtained with DPPH assay varied from 5.12±0.34 to 14.17±0.32 μg mL^{-1}. Total phenolic varied from 214.32±0.33 to 510±0.21 μg mL^{-1} and flavonoid content, from 15.26±0.63 to 39.92±0.03 μg mL^{-1}. The species *C. domestica* exhibited high antioxidant potential, being ethyl acetate the most active fraction. Several bioactive phenolics were identified and quantified in this fraction. All compounds are described for the first time for the genus *Cariniana*.

Key words: DPPH, free radicals, Folin Ciocalteau, phytochemisty, polyphenols

INTRODUCTION

The species *Cariniana domestica*, commonly known in Brazil as jequitibá-roxo belongs to Lecythidaceae, a family consisting of 25 genera and 400 species that occur in the form of large trees, with pantropical distribution (Tsou and Mori, 2007). Several important bioactive compounds have been isolated from species of this family and therefore, it is important for the conduction of further studies on this genus (De Carvalho *et al.*, 1998; Lima *et al.*, 2002). There are few reports in the literature concerning to chemical constituents and ethnopharmacological uses of the genus *Cariniana*, although the stem bark and the leaves infusion of these species are commonly used as anti-inflammatory and antimicrobial remedies (Silva *et al.*, 2009).

Previously, Lima *et al.* (2002) has isolated triterpenoidal saponins from the barks of *Cariniana rubra* Gardner and Miers whereas some constituents have been isolated from other species of this family, including pentacyclic triterpenes, saponins, ellagic acid and indol-quinazoline alkaloids (De Carvalho *et al.*, 1998). Further works related to *Cariniana* spp. describe the species as skin-lightening cosmetic by inhibition of hyaluronidase and also shown anti-tyrosinase activity (Baurin *et al.*, 2002) and also as antifungal (Silva *et al.*, 2009). A previous work from our group

led to the isolation, identification and quantification of four bioactive substances from the dichloromethane fraction obtained with the barks of *C. domestica* (β-sitosterol, stigmasterol, lupeol and β-amyrin) (Janovik *et al.*, 2011).

Several authors have researched different extracts of plants in order to improve knowledge about antioxidant capacity. According to Kintzios *et al.* (2010), it is important to identify the compounds involved in this capacity as well as to evaluate the free radical-scavenging from crude extracts because it is the common form of usage in popular medicine. Polyphenolic compounds have been largely studied as antioxidant compounds and its dietary ingest have shown protective effect against diseases such as coronary heart (Engler and Engler, 2006) and neurodegenerative (Lau *et al.*, 2005).

Preliminary phytochemical investigations of our group concerning the leaves of *C. domestica* indicated the presence of a large number of phenolic compounds. This feature allied to the importance of the oxidative stress in the pathogenesis of various diseases prompts us to better evaluate the antioxidant potential of this plant and to determine the phenolic and flavonoid contents. Simultaneously, HPLC/DAD was performed to identify which were the mainly phenolic compounds and to quantify them.

MATERIALS AND METHODS

Reagents, standards and apparatus: Silica Gel 60, Silica Gel 60 F254 coated plates, solvents for the extractions, dichloromethane, ethyl acetate, ethanol, methanol and n-butanol, Folin-Ciocalteau and aluminum chloride were purchased from Merck (Darmstadt, Germany). Ascorbic acid, 2,2-diphenyl,1-picrylhydrasyl (DPPH), quercetin, rutin, kaempferol, gallic, chlorogenic and caffeic acids standards were acquired from Sigma Chemical Co. (St. Louis, MO, USA). 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. Spectrophotometer was Shimadzu-UV-1201.

Plant collection and extraction: The leaves 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 in a knife mill. The powder was macerated with ethanol 70% 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 after ethanol evaporation was partitioned with solvents in increasing polarity to obtain the following fractions: dichloromethane, ethyl acetate and n-butanol.

Radical-scavenging activity-DPPH assay: The antioxidant activity of the fractions and crude extract was evaluated by monitoring its ability in quenching the stable free radical DPPH, according to a slightly modified method previously described (Choi *et al.*, 2002). DPPH quenching ability was expressed as IC_{50}. Six different ethanol dilutions of each fraction and crude extracts at 250, 125, 62.5, 31.25, 15.62 and 7.81 μg 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. Ethanol was used to

calibrate the spectrophotometer. The test was performed in triplicate and the calculation of the antioxidant activity followed the equation:

$$\% \text{ inibition} = 100 - \frac{[(\text{AbsS} - \text{AbsB}) \times 100]}{\text{AbsC}}$$

where, AbsSis the absorbance of each fraction; AbsB is the absorbance of fractions without adding the DPPH; AbsC is absorbance the ethanolic solution of 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 contents was performed by the Folin-Ciocalteu method (Chandra and Mejia, 2004). The total phenolic content was expressed in milligrams equivalents of pyrogallic acid (PGA) per gram of each fraction. The equation obtained for the calibration curve of pyrogallic acid in the range of 0.005 to 0.030 mg mL^{-1} was y = 34.443x-0.0942 (r = 0.9984).

Determination of total flavonoids: The determination of flavonoid content was performed using the method described by Woisky and Salatino (1998). Total flavonoid content was expressed in milligrams equivalents of rutin (RT) per gram of each fraction. The equation obtained for the calibration curve of rutin was Y = 20.394 x-0.2033 (r = 0.9997).

HPLC-DAD qualitative and quantitative analysis of polyphenols: Reverse phase chromatography analyses were carried out with a Phenomenex C-18 column (4.6 mm x 250 mm) packed with 5 μm diameter particles, volume injection was 40 μL and the gradient elution was conducted according to the Evaristo and Leitao (2001) method slightly modified. 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 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 μm membrane filter (Millipore). The chromatographic operations were carried out at ambient temperature and in triplicate.

Statistical analysis: Correlation coefficient (r) was used to determine the relationship between two variables and the standard deviations in the DPPH, total phenolic and total flavonoid assays were calculated from the data obtained from three separate experiments using MS Excel for Windows.

RESULTS AND DISCUSSION

The phenolic substances such as tocopherols, flavonoids and phenolic acids are specially mentioned as antioxidants due to their efficient scavenging capacity of reactive oxygen species which may cause a large number of disorders by reacting with cellular lipids, proteins, carbohydrates and nucleic acids (Chanwitheesuk *et al.*, 2005). Besides, natural antioxidants pursuits better benefits in comparison to synthetic ones (Stankovic *et al.*, 2010).

In this study, the leaves of *C. domestica* exhibited very good antioxidant activity, especially ethyl acetate fraction. The results are shown in Table 1.

Ethyl acetate was the most active fraction. The IC_{50} (the amount of extract of the plant tested necessary to decrease the concentration of initial DPPH absorbance by 50%) value obtained was 5.12±0.34 µg mL^{-1} (Table 1). Butanolic fraction showed slightly lower IC_{50} value (5.73±0.81 µg mL^{-1}) than ethyl acetate fraction, being also very active and the crude extract IC_{50} was 6.95±0.62 µg mL^{-1}. The lowest antioxidant activity (IC_{50} = 14.17±0.32 µg mL^{-1}) was observed for dichloromethane fraction. Ethyl acetate, butanolic and crude extract antioxidant activities were found to be higher than the well known antioxidant ascorbic acid, commonly used as a standard (IC_{50} = 9.02±0.21 µg mL^{-1}).

Previous work has found similar good results for ethyl acetate and butanolic fractions and have documented that these fractions are sources of antioxidant substances (Tung *et al*., 2007). Antioxidant compounds of different chemical structure can be achieved using solvents of different polarity (Stankovic *et al*., 2010) and some investigations of plants compositions have demonstrated that polar fractions usually concentrates high amounts of phenolic compounds (Canadanovic-Brunet *et al*., 2008).

It is important to compare data obtained on *C. domestica* with other plants under the same assay conditions (Schubert *et al*., 2007). If compared with green tea (*Camellia sinensis*), the results obtained with *C. domestica* were better than those obtained for this plant, IC_{50} = 11.8 µg mL^{-1} (Almeida *et al*., 2008). *Ilex paraguariensis*, a popular species from South Brazil which achieves antioxidant properties, have exhibited superior values for ethyl acetate fraction (IC_{50} = 13.26 µg mL^{-1}) when compared with *C. domestica* (Schubert *et al*., 2007).

For the determination of phenolic content, the ethyl acetate fraction also exhibited the highest value (510±0.21) (Table 1). Butanolic fraction expressed the second highest value (486.22±0.87). On the other hand, dichloromethane fraction showed the lowest phenolic content (214.32±0.33). When comparing the results obtained with phenolic and flavonoid contents assays, we may observe a correlation between both dosages for ethyl acetate fraction. Differently, butanolic fraction, despite presenting high levels of phenolic compounds, ranged a low value in the flavonoid determination. Dichloromethane fraction exhibited a high content of flavonoids (38.01±0.1 mg g^{-1}), being the second best value. On the other hand, the value obtained for total phenolic content was the lowest (214.32±0.33 mg g^{-1}).

Ethyl acetate fraction furnished high concentrations of polyphenols and flavonoids, as well as the best IC_{50} value in DPPH assay. Therefore, we have performed an HPLC/DAD analysis with the purpose of identify and quantify its mainly phenolic compounds.

Table 1: Total phenolics and flavonoid contents and antioxidant activities (IC_{50}/DPPH) from the leaves of *C. domestica*

	Fraction		
Extract	Total phenolic (mg PGA/g)	Total flavonoids (mg RT/g)	IC_{50} (µg mL^{-1})
Dichloromethane	214.32±0.33	38.01±0.10	14.17±0.32
Ethyl acetate	510.00±0.21	39.92±0.03	5.12±0.34
Butanol	486.22±0.87	15.26±0.63	5.73±0.81
Crude extract	480.03±0.25	28.65±0.09	6.95±0.62
Ascorbic acid	-	-	9.02±0.21

Values are as Mean±SE. PGA: Pyrogallic acid, RT: Rutin, SE: Standard error, IC_{50} = The amount of extract necessary to decrease the concentration of initial DPPH absorbance in 50%

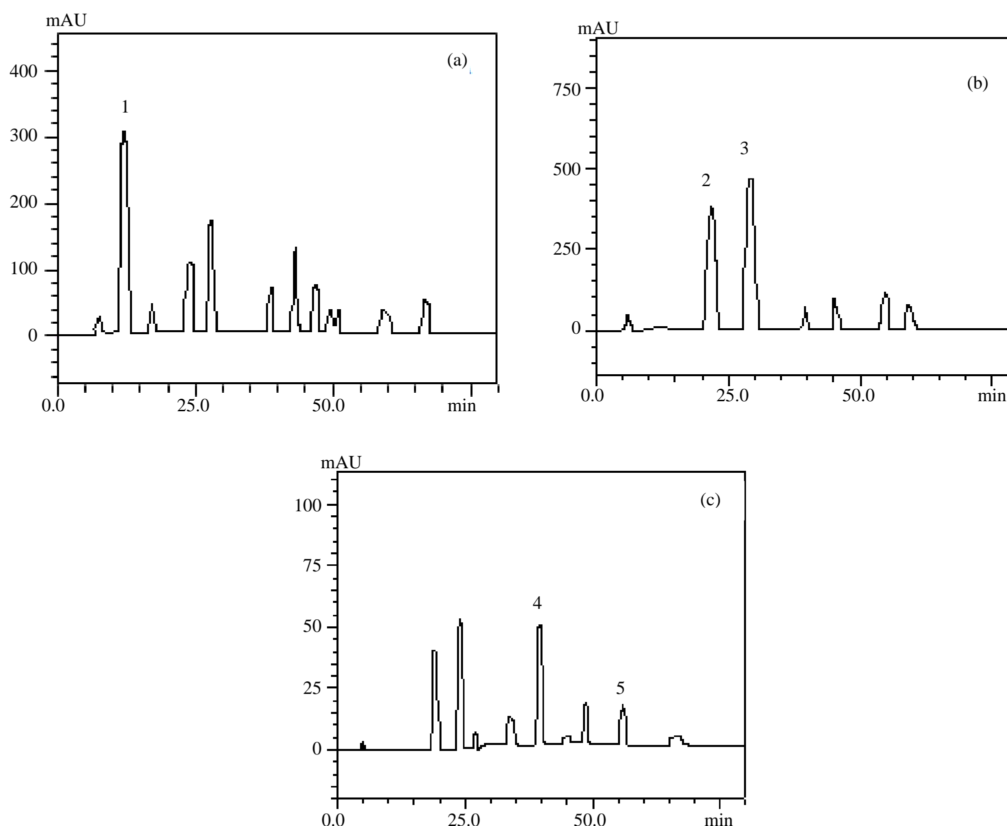

Fig. 1: HPLC/DAD chromatograms obtained with ethyl acetate fraction. a = ethyl acetate, 254 nm; b = ethyl acetate, 327 nm; c = ethyl acetate, 365 nm. Peaks: 1 = gallic acid (RT = 12.8 min); 2 = chlorogenic acid (RT = 24.6 min); 3 = caffeic acid (25.8 min); 4 = rutin (RT = 38.2 min); 5 = kaempferol (RT = 55.1 min)

Table 2: HPLC/DAD of identified and quantified phenolic compounds in ethyl acetate fraction obtained from the leaves of *C. domestica*

	mg g^{-1} of dried fraction[a]					
Fraction	GA	CLA	CFA	RT	QT	KF
Ethyl acetate	2.50±0.82	15.27±1.21	12.82±0.93	11.45±0.32	-	0.85±1.11

[a]Results are expressed as Mean (n = 3) ±SD. G: Gallic acid; CFA: Caffeic acid; CLA: Chlorogenic acid; RT: Rutin; QT: Quercetin; KF: Kaempferol

Ethyl acetate fraction was investigated for the presence of the following compounds: gallic, chlorogenic and caffeic acids, quercetin, rutin and kaempferol. Identification was performed by comparison of their retention's time and UV absorption spectrum with those of the standards.

The results and chromatograms are shown in Table 2 and Fig. 1. All tested substances are well-known antioxidants. Gallic acid is a derivative from benzoic acid widely distributed in various plants and foods and its various biological effects have been reported, such as anti-proliferative, pro-apoptotic and anti-tumorigenic effects against prostate (Kaur *et al*., 2009). In this work, gallic acid was found in minor quantities in ethyl acetate fraction (2.5±0.82%).

Caffeic and chlorogenic acids have been reported as good free radical scavengers (Arrua *et al.*, 2010). Our analysis revealed that caffeic acid constitutes 12.82±0.93% of whole ethyl acetate fraction. In the HPLC/DAD analysis, chlorogenic acid was found in major quantities (15.27±1.21%).

Rutin (quercetin 3-O-rhamnoglucoside) which is commonly found in plants, upon the hydrolysis of the glycoside bond produces quercetin, a highly antioxidative aglycon. This flavonoid is an active and natural antioxidant, involved in protection and prevention of pathologies like diabetes mellitus, as an example (Fernandes *et al.*, 2010). In this analysis, rutin constitutes 11.45±0.32% of whole fraction.

Finally, kaempferol, a flavonol with a free hydroxyl group at the C-3 position, represented 0.85±1.11% from ethyl acetate fraction.

CONCLUSIONS

Values for antioxidant activity and concentrations of phenolic and flavonoid contents obtained for crude extract and fractions from *C. domestica* exhibited positive correlations. Ethyl acetate fraction ranged the highest flavonoid and phenolic content and this significant correlation indicates that these compounds contribute to the antioxidant capacity. All results obtained are reported for the first time for *C. domestica*.

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