Voltammetric and spectrophotometric determination of antioxidant activity of *Eugenia dysenterica* DC leaves extracts

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Abstract: *Eugenia dysenterica* DC (cagaiteira) is a native tree from Cerrado biome. Cagaita fruits are known and consumed *in natura*, mainly by inhabitants from Cerrado. In this study, we evaluated the antioxidant activity of leaves of this plant. For this evaluation we used four methods, the reduction of phosphomolybdenum, scanning by hydrogen peroxide, DPPH radical scavenging assay and determination of electrochemical parameters by differential pulse voltammetry. The results indicate that all extracts from leaves of this species have significant antioxidant potential, following the order: crude ethanol extract CEE) >crude aqueous extract (CAE) >crude hexane extract (CHE). The voltammetric approaches were also applied in order to evaluate the redox behavior of the hydrophilic extracts, as well as of their sub-extracts. Thus, the results suggest the presence of catechol-like polyphenols, which were confirmed by chromatography and phytochemical methods. Voltammetric analysis showed to be a reliable and fast method to determine redox behavior of *E. dysenterica* extracts.

Keywords: *Eugenia dysenterica*, Antioxidant activity, differential pulse voltammetry.

INTRODUCTION

Eugenia dysenterica DC. (Myrtaceae), popularly known as cagaiteira is a native tree in Cerrado biome(Lorenzi *et al.*, 2006). This species is found in regions with annual average temperatures between 21ºC and 25°C and situated in altitude of 380 to 1100 meters (Naves, 1999, Souza *et al.*, 2008). Also, because cagaiteira is a flowering tree, this species can be found in home gardens of small-scale farmers (Duque-Brasil *et al.*, 2012, Tunholi, 2011). The flowering period occurs between July and August, corresponding to dry season (Brito *et al.*, 2003).The leaves are membranous, opposites, oblongs, simples, short petiolate, glabrous (Palhares, 2003). They also have slightly acuminate apex; obtuse base ranging from the subcordata reticulated venation and marginal veins not forming sharp(Rizzini, 1970). Pharmacognostic characterization and physical chemistry studies of the leaf powder showed that the plant is rich in phenolic compounds, flavonoids, tannins, saponins and terpenes (Couto *et al.*, 2009).

Cerrado inhabitants use the edible cagaita fruits *in natura* or to prepare drinks, juices, and desserts. This species, besides the ethnobotanical relevance, also presents economic importance. The fruits have been industrially processed to obtain different types of sweets, popsicles, wine, and ice cream (Vieira *et al.*, 2012). As for its medicinal use, cagaita fruits present laxative effect, while leaves have therapeutic benefits on recovery from diarrhea (Agra *et al.*, 2008, Lima *et al.*, 2010). They are also used to treat heart diseases (Brandão, 1991, Conceição *et al.*, 2012), diabetes and jaundices (Lima *et al.*, 2011).

Previous studies have shown that ethanol extract from fruits of cagaita has the capacity to sequester free radicals (Roesler *et al.*, 2007, Oliveira *et al.*, 2012, Souza *et al.*, 2012, Almeida Siqueira *et al.*, 2013). Indeed, such activity is related to the presence of polyphenol compounds (Couto *et al.*, 2009, Almeida Siqueira *et al.*, 2013).

Concerning *E. dysenterica* leaves, they present inhibitory activity against *Staphylococcus aureus*, as well as antiulcer activity with results statistically comparable to ranitidine(Junqueira, 2007). Despite hydroalcoholic extract showed cytotoxic and genotoxicity in high doses, the ethanol extract decreased intestinal motility, confirming the ethnomedicinal use as anti-diarrhoea remedy (Vieira *et al.*, 2012). In addition, the ethanol, aqueous and hexane extracts from leaves were able to inhibit the tyrosinase activity with activity comparable tokojic acid (Souza *et al.*, 2012). In turn, taking into account the cost-effectiveness and chemical diversity of plant extracts, these sources of natural antioxidants have been attracted the interest of pharmaceutical and cosmetics sector (Jayaprakasha and Rao, 2000).

Thus, the aim of this work was to evaluate the antioxidant activity of leaves extracts of *Eugenia dysenterica*, considering that to the best of our knowledge, no previous

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study on the antioxidant activity has been performed for them.

MATERIAL AND METHODS

Plant material

Leaves of *Eugenia dysenterica* DC were collected at the University of Brasilia, Campus Darcy Ribeiroin Brasilia-DF, Brazil. A voucher species was deposited at Universidade de Brasilia Herbarium (UB 914). The plant material was cleaned with water and then was dried in an oven. The dried leaves were selected in order to remove any remained dirt and pulverized in a blender then be subjected to extraction.

Part of plant material was submitted to decoction in distilled water at ratio of 1:10 (leaf: water). The aqueous extract (CAE) was filtered, cooled and then lyophilized. For organic extracts, leaves of *E. dysenterica* DC were macerated using different solvents, hexane, and ethanol in a ratio of 1: 10 (leaf: organic solvent). After filtration, the solvents were eliminated under vacuum and temperature below 40°C to furnish hexane (CHE) and ethanol (CEE) crude extracts. The obtained crude extracts were stored at low temperature until be use din subsequent trials.

Determination of antioxidant activity

Reductive activity of molybdenum VI

The antioxidant activity was evaluated by the phosphomolybdenum method according to the procedure previously described (Prieto *et al.*, 1999). Aqueous solution of extracts was obtained at concentration of 1mg/mL. An aliquot of 0.1mL of the obtain solution was added to 1mL of the reagent solution. The mixture was incubated in a water bath (Grant Y28) at 95°C for 90min. After cooling, the absorbance of each sample was measured at 695 nm in a spectrophotometer (Shimadzu® UV-1601/UV-visible) against a blank incubated under the same conditions. Standard curves were constructed with µM solutions of quercetin, BHT (2,6-bis(1,1-dimethylethyl)-4-methylphenol) and ascorbic acid under the conditions listed above. The antioxidant capacity of the sample was expressed as equivalents of quercetin, BHT and ascorbic acid per g of extract (Prieto *et al.*, 1999).

Scanning method for hydrogen peroxide

Scanning method for hydrogen peroxide was also employed to evaluate the antioxidant activity of all extracts herein prepared, following the procedures previously described (Ruch *et al.*, 1989). Briefly, a solution of hydrogen peroxide (40mM) was prepared in phosphate buffer (pH 7.4). The concentration of hydrogen peroxide was determined by absorption at 230nm using a spectrophotometer. The ability of the samples and standard (25 to 1000µg.mL^{-1}) to scavenge free radicals from hydrogen peroxide was evaluated and compared.

In each experiment, 680 µL of buffer and 120 µL of hydrogen peroxide were added to 200 µL of extract (or standard). The mixture was incubated for 10 minutes and then the absorbance of each sample was measured at 230 nm in a spectrophotometer. Calibration curves were constructed for ascorbic acid (positive control), and each extract. The absorbance of hydrogen per oxide at 230 nm was determined after ten minutes against a blank solution containing phosphate buffer without hydrogen peroxide. The percentage of hydrogen peroxide scavenging by the extracts and standard compounds were calculated as follows (Equation 1):

$$\% \text{ Scavenged } [H_2O_2] = [(A_0 - A_1) / A_0] \times 100$$

Where A_0 was the absorbance of the control and A_1 was the absorbance in the presence of the sample of extract or standard. EC_{50} was determined by the required amount of sample or standard to reduce by 50% the hydrogen peroxide absorption.

DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity

DPPH radical scavenging activity assay was performed according to the literature(Yildirim *et al.*, 2001). DPPH reagent was prepared (c.a. 0.004%) to obtain an absorbance of 0.70±0.05 at 517nm. Briefly, 4mL of the DPPH solution prepared as described above, were taken, divided into two beakers, one for testing and another for the negative control. In the remainder part of the initial solution were added 4mL of ethanol, to obtain a solution with 60% concentration of the original solution. The procedure was repeated for six times, to obtain seven solutions with successive dilutions in decreasing concentrations from 200 to 10ppm. For each test sample was added 1mL of DPPH. For each negative control, was added 1mL of ethanol, in order to maintain the same concentration of crude extract in the sample and the negative control. After the addition of DPPH solution, the samples were protected from light for 30 minutes. The antioxidant activity of the solutions was assessed by measuring the absorbance decay at 517 nm in a spectrophotometer. The antioxidant activity for each concentration was achieved by the Equation 2:

$$\% \text{ Antioxidant activity} = [100- (\text{sample} - \text{blank}) \times 100] / \text{control}$$

For the calculation of EC_{50}, it assumed the amount of extract or standard to lead 50% of the decolorization.

Electro analytical assays

Voltammetric experiments were performed in a potentiostat/galvanostat µ Auto lab III® integrated to the GPES 4.9® software, Eco-Chemie, Utrecht, The Netherlands. Measurements were performed in a 5.0 mL one-compartment electrochemical cell, with a three-electrode system consisting of a carbon paste electrode

Table 1: Equivalence of standards compared to *E. dysenterica* DC extracts

Samples			Standard Equivalence (mg.mL^{-1})		
Extract	Concentration	ABS	Quercetin	Ascorbic Acid	BHT
CEE	1 mg. mL $^{-1}$	1.171	1.255	1.193	0.777
CHE		0.547	0.631	0.569	0.153
CAE		0.577	0.661	0.599	0.183

Table 2: Antioxidant capacity of *E. dysenterica* DC extracts, by scanning method for Hydrogen Peroxide.

Extract	EC_{50} (*m*g.mL^{-1})		Linear Correlation
	Extracts Ascorbic Acid		
CEE	272.95	274.12	0.82
CHE	273.83		0.82
CAE	273.78		0.82

Table 3: Antioxidant capacity of *E. dysenterica* DC extracts, by DPPH radical scavenger method.

Extract	EC_{50} (*m*g.mL^{-1})		Linear Correlation
	Extract BHT		
CEE	210.58	211.79	0.80
CHE	210.85		0.80
CAE	210.21		0.80

Table 4: Statistical test T Student - One-Sample Test results for antioxidant activity of *E. dysenterica* DC extracts

Test Value = 0						
Extracts	T	N	Sig.(2-Tailed)	Mean Difference	Lower	Upper
CEE	2.926	5	0.033	0.319	0.038	0.60
CHE	3.715	5	0.014	0.274	0.084	0.46
CAE	2.889	5	0.034	0.305	0.033	0.57

CEE - crude ethanol extract; CHE - crude hexane extract; CAE - crude aqueous extract

(prepared as a piston-driven holder containing 70% of graphite powder and 30% of Nujol®, Ø =2mm), a Pt wire and the Ag/AgCl/KCl_{sat} (both purchased from Analyser, São Paulo), representing the working electrode, the counter electrode and the reference electrode respectively.

The surface of the carbon paste electrode (CPE) was mechanically renewed before the start of a new set of experiments by extruding approximately 0.5mm of carbon paste out of the electrode holder and smoothing it with filter paper. This procedure ensured very reproducible experimental results.

The experimental conditions for differential pulse voltammetry (DPV) were: pulse amplitude 50mV, step potential 4.5mVs^{-1} width 0.4 s and scan rate 10mV s^{-1}. For square wave (SWV) voltammetry were: pulse of 20mV, frequency of 15 Hz and a potential increment of 4.5mV, corresponding to an effective scan rate of 55 mVs^{-1} were used. All experiments were done at room temperature (21±1ºC) in triplicate (*n=3*).

Electrochemical index determination

An Electrochemical Index (EI) was achieved as previously described (Lino, 2013). Briefly, the sum of the rates for the main voltammetric parameters, peak potential (E_{pa}) and peak current (I_{pa}) was obtained for all extracts by applying the Equation 3.

$$EI = \frac{I_{pa1}}{E_{pa1}} + \frac{I_{pa2}}{E_{pa2}} + \cdots + \frac{I_{pan}}{E_{pan}} \quad (1)$$

In which I_{pa} and E_{pa} correspond to current and potential value for each major anodic peak observed in the DPV voltammograms. The statistical test T Student with the program SPSS - Statistical Package for Social Sciences (version 18.0) was applied.

RESULTS

The antioxidant activity of the samples were evaluated according to their ability to reduce molybdenum (VI) leading to a green molybdenum (V) complex, which has an absorption at 695 nm. In our experiments, all samples caused the reduction of molybdenum, and the ethanol extract showed higher reducing power (table 1).

The ability to sequester free radicals from hydrogen peroxide in comparison with ascorbic acid was also evaluated for CHE, CEE and CAE. The results are presented in table 2.

The higher antioxidant capacity, expressed by the lower EC_{50} values, followed the order CEE > CAE > CHE, respectively.

The DPPH assay is one of the most traditional methods applied to the antioxidant activity evaluation. Therefore, such method is useful to establish comparisons with other subjects of study, and the results are presented in table 3.

Fig. 1A shows the cyclic voltammogram obtained for different hydrophilic extracts. It was found for all hydrophilic extracts the presence of four oxidation peaks, 1a, 2a, 3a and 4a, at peak potentials E_{pa} of 0.28, 0.58, 0.78 and 0.97 V respectively. On the reverse scan, it was observed two cathodic peaks, 1c and 2c, at E_{pc} of 0.20 and -0.13 V respectively. Such pattern is in agreement with the presence of a number of phenolic compounds /groups (Couto *et al*., 2009, Gil and Couto, 2013).

In order to improve the resolution of anodic peaks, the DPV voltammetry was performed. Aside the expected shifting to lower potentials, varying from 50 mV for peaks, 1a and 2a and of 150 mV for peak, 3a, similar results can be observed in DPV voltammograms, showed at fig. 1B.

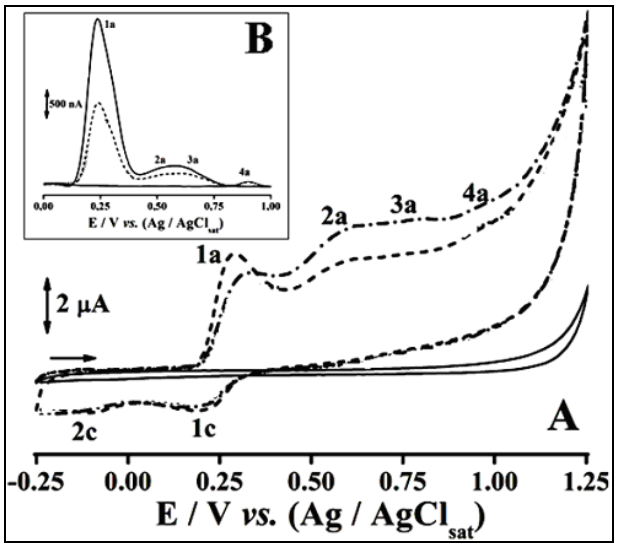

Fig. 1: (A) Cyclic Voltammetry voltammograms for blank (——) and *E. dysenterica* DC extracts from methanol (— — —), ethanol 10% (·····) and acetone (— · —) at rate scan of 100mVs^{-1} and step potential of 12 mV. (B) Differential Pulse Voltammetry voltammograms for same extracts at scan rate 20 mVs^{-1}, step potential 4.5 mV and modulation amplitude of 50 mV. For measurements 5 µL of each extract was used in 10ml of 0.1M phosphate electrolyte solution at pH 5.

Though the peak potentials obtained for all extracts remained unchanged, the peak currents varied according to the extraction solvent system employed, being higher according to the polarity. On the other hand, owing to the good repeatability of electroactive surface area of carbon paste electrodes, the peak currents can be also compared.

Hence, by applying the equation 1, the obtained in order, the higher peak current levels were obtained for ethanol extract, whereas the lower was achieved for less hydrophilic extract.
electrochemical index for the ethanol, aqueous and hexane extracts were 13.6, 7.7 and 5.4 µA/V, respectively, which is in agreement with the results obtained by the traditional spectrometric methods (Lino *et al.*, 2013).

SWV voltammetry was also applied to the electrochemical characterization of hydrophilic extracts. In SWV voltammetry, the current is sampled in both positive and negative-going pulses. Hence, both the oxidation and reduction peaks can be obtained simultaneously at the electrode surface and the reversibility of the electron transfer reaction monitored by plotting the forward and backward components leads to higher total current and sensitivity.

Fig. 2 presents the SWV voltammograms obtained for ethanol extract, in which can be observed better resolution for the quasi-reversible redox process 1a/1c.

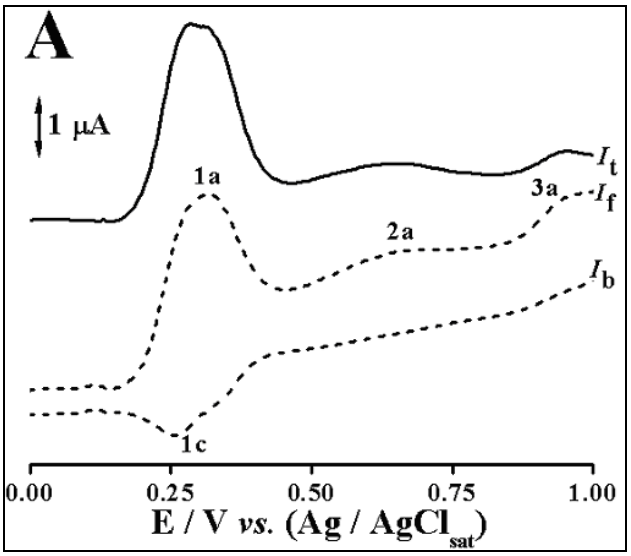

Fig. 2: Square wave voltammograms obtained for 50µL of *E. dysenterica* DC ethanol crude extract (CEE) in 5mL of pH 7.0 0.1 M Phosphate buffer.

The same behavior was observed for the aqueous extract. It must be noted that it is important to find the most suitable solvent, in order to obtain selective extraction of natural antioxidants. Additional studies will be needed for the steps of isolation, characterization of compounds responsible for antioxidant activity and, finally, to elucidate the mechanism of action of these compounds.

DISCUSSIONS

According to table 1, the reducing power of ethanol extract was twice higher than the other extracts. The obtained values (table 2) confirmed that the three extracts can scavenge free radicals from hydrogen peroxide, indicating their antioxidant capacity.

Though the achieved order is the same of the one obtained by phosphomolybdenum method, the obtained values presented similar magnitude. Also, according to the EC_{50} values, it can be inferred that the extracts possess higher or comparable antioxidant activity than the ascorbic acid, used as positive control.

Therefore, it can be assumed that *E. dysenterica* leaves extracts are a very powerful source of natural antioxidants.

The antioxidant profile exhibited in table 3 for DPPH assay is closer to that one observed in table 2. In fact, both methods are related to radical scavenging activity. On the other hand, in the case of DPPH radical assay, lower EC_{50} values were obtained for aqueous extracts.

Also BHT, positive control, showed higher EC_{50} values than the obtained for all samples. Moreover, the electrochemical measurements have reinforced the antioxidant content in *E. desinterica* leaves extracts. Indeed, the peak current is a kinetic parameter that expresses the amount of electro active species, *i.e.* antioxidants and also the speed in which they transfer electrons. Furthermore, from the low peak potentials obtained for all extracts (E_{pa} <0.6 V), it can be assumed that the antioxidant species have great reducing capacity. Indeed, the peak potential is an electrochemical parameter that expresses the electron donor character. Thus, lower is the peak potential, higher is the thermodynamic strength in which a compound or chemical group undergoes oxidation process, and higher is its reducing power.

Another, relevant characteristic that was taken from electrochemical assays is related to the reversible behavior observed in SWV experiments (Fig. 2). In fact, the redox reversibility expresses the capacity to regenerate and is usually attributed to the best antioxidants. Such behavior indicates the presence of catechol-like phenolic compounds, recognized by higher electron donor character and antioxidant properties (Gil and Couto, 2013).

Therefore, the antioxidant activity of extracts can be attributed to the ability of some substances in the plant to scavenge free radicals by donating hydrogen. Therefore, such results indicate the presence of compounds with significant antioxidant activity of extracts from the leaves of *Eugenia dysenterica*.

CONCLUSIONS

As far we know, this is the first study that reports the antioxidant activity of leaves extracts from *E. dysenterica* DC. Compared to the data obtained under experimental conditions used, it is concluded that the three extracts tested possess antioxidant activity. The best antioxidant activity was observed for ethanol extracts, which presented remarkable reductive activity of molybdenum and slightly higher DPPH radical scavenging activity, respectively. On the other hand, the aqueous extracts exhibited similar, but higher performance, when submitted to the scanning method for hydrogen peroxide. In order the Cyclic and DP voltammetric assays allow the tentative identification of redox-active compounds, which may present antioxidant or pro-oxidant activity. Thus, the voltammetric profile suggests the presence of phenolic compounds with variable electro-active moieties. The SW voltammetry have indicated the presence of catechol-like compounds, *i.e.* flavonoids, recognized as powerful natural antioxidants. Therefore, the obtained results suggest *E. dysenterica* extracts that may bea useful source of antioxidant compounds.

Indeed, it is in agreement with previous work, in which was found the presence of flavonoids, catechins and other phenolic compounds, as well as terpenes (Cecílio *et al.*, 2012, Souza *et al*., 2012).

Moreover, the antioxidant assays on different extracts have indicated that the correlation between total phenolics and antioxidant capacity may depend not only on the solvent employed on the extraction process, but also on the method employed on the antioxidant evaluation assay (Gil and Couto, 2013). Applying the statistical test T Student with the program SPSS - Statistical Package for Social Sciences (version 18.0), it was found that the ethanol extract showed the best antioxidant activity, with $p = 0.033$ (table 4).

Since ethanol and aqueous extracts have presented higher antioxidant activity, they were submitted to electrochemical characterization, in order to estimate their redox profile.

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