

Identification of catechin in ethyl acetate phase and determination of redox profile of the *Anacardium occidentale* L. through electrochemical techniques

Identificação da Catequina da fase acetato de etila e determinação do perfil redox da *Anacardium* occidentale L. através de técnicas eletroquímicas

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Anacardium occidentale L., also known how "cajueiro", is very used for your pharmacological properties, like: anti-inflammatory, healing, anti-glycemic and others. Their curls have been described as rich in phenolic compounds, powerful natural antioxidants. Thus, the objective of this work was to determine the redox profile of the A. occidentale inner bark extract using electrochemical and spectrophotometric techniques through the DPPH radical sequestration reaction. For this, a phytochemical prospection was made in which steroids, phenols, flavones, flavonoids, saponins, tannins and xanthones were detected in the hydroethanolic extract (HEE) and in the ethyl acetate phase (EAP). Total phenolic content in the EAP was also quantified with values of 848.62 \pm 78.18 µg mg⁻¹ of gallic acid. The DPPH assay showed that the EAP at a concentration of $25\mu g$ mL⁻¹, presents the greater sequestration of free radicals (around 90%) with an CE₅₀ value of 16.13 μg mL⁻¹ and an AAI value of 2.48, noting the very strong antioxidant potential of the plant. The EAP High Performance Liquid Chromatography analysis showed a similar behavior to the catechin standard, with a major peak eluted at 3 minutes and 30 seconds with a slightly broad base and a UV absorbance spectrum with a maximum absorbance of 274 nm, identifying (+) - catechin, when compared to the 279 nm UV-Visible scanning spectrum obtained with catechin standard. The EAP voltammograms were similar to the (+) catechin standard, inferring the catechin involvement with A. occidentale redox activity. Keywords: antioxidant activity, electrochemistry, catechin

Anacardium occidentale L., o popular cajueiro, é muito conhecido por suas propriedades farmacológicas, tais como: anti-inflamatória, cicatrizante, antiglicêmica e afins. Suas entrecascas foram descritas como ricas em compostos fenólicos, poderosos antioxidantes naturais. Assim, o objetivo deste trabalho foi determinar o perfil redox do extrato das entrecascas de A. occidentale utilizando técnicas eletroquímicas e espectrofotométricas por meio da reação de sequestro do radical DPPH. Para isso, foi feita uma prospecção fitoquímica em que foram detectados esteroides, fenóis, flavonas, flavonoides, saponinas, taninos e xantonas no extrato hidroetanólico (HEE) e na fase acetato de etila (EAP). Foi quantificado o teor de fenólicos totais na EAP com valores de 848,62 \pm 78,18 μ g mg⁻¹ de ácido gálico. O ensaio DPPH mostrou que na concentração da EAP de 25 µg mL⁻¹ foi onde houve o maior sequestro do radical livre (em torno de 90%) com valor de CE_{50} de 16,13 µg mL⁻¹ e com valor de AAI 2,48, constatando o potencial antioxidante muito forte da planta. A análise por Cromatografia Líquida de Alta Eficiência da EAP apresentou um comportamento similiar ao padrão catequina, com um pico majoritário eluído em 3 minutos e 30 segundos com base pouco alargada e com um espectro de varredura UV com máximo de absorbância de 274 nm, e assim, identificando a (+) catequina, ao comparar com o espectro de varredura UV-visível de 278 nm obtido com padrão de catequina. Os voltamogramas da EAP apresentaram semelhança com o padrão (+) - catequina, inferindo no envolvimento da catequina com a atividade redox da A. occidentale. Palavras-chave: atividade antioxidante, eletroquímica, catequina

1. INTRODUCTION

Since the beginning, human beings have made use of the natural properties of plants, in the treatment and cure of diseases [1]. Many botanical families have been promising for bioactive substances, including Anacardiaceae [2]. The species in this family stand out for their diversity of use, ranging from edible fruit production to use in popular medicine [3].

Brazil has a huge variety of fruit species, especially the northeast region [4]. Plants are largely responsible for the diversity of substances in their various applications, mainly in the treatment of diseases. Thus, they represent a large reservoir of valuable molecules to be discovered [5, 6].

Among the most exploited species is *Anacardium occidentale* L., known as cashew tree, from which many chemical compounds have been isolated, identified and its potential has been evaluated and used [7]. The presence of polyphenolic compounds in this species has been reported, as well as the relationship of this class of secondary metabolites with antioxidant activity [8, 9].

Phenolic compounds are not harmful to human health and have defensive and disease-fighting properties [8, 10]. The antioxidant activity of polyphenols can play an important role in preventing oxidation and protecting against DNA damage by stabilizing or disabling free radicals before they attack biological targets in cells. In addition, these compounds have various biological activities, such as anti-inflammatory and anticarcinogenic activity [11].

Currently, the antioxidant activity of plant derivatives has been evaluated by different methods, such as calorimetry, biological and electrochemical. A correlation between techniques such as electrochemical and free radical capacity (DPPH) can contribute and enrich species antioxidant profile analyzes [12].

Electrochemical techniques are applied to the study of electroactive species, whether isolated substances or extracts of plants, herbal medicines or other compounds commonly used as antioxidants. They have great potential for detailed characterization of phytoantioxidants as they provide physicochemical parameters capable of showing beyond the redox potential, the electron numbers involved, proton influence, reaction constants, etc. [13].

Voltammetric techniques correlate oxidation potentials, current intensity and/or other electrochemical parameters with antioxidant capacity, and are more selective and sensitive than other spectrometric methods for the evaluation of antioxidant activity, as well as more reproducible than biological analyzes [13-15]. Moreover, they are an important tool for the analysis of organic and inorganic compounds.

Therefore, in this work were performed the phytochemical prospecting of the hydroethanolic extract (HEE) and the ethyl acetate phase (EAP) of the *Anacardium occidentale* inner bark and evaluated the redox activity of the EAP through the DPPH free radical and electrochemical techniques, and seek to relate them to catechin (CAT) expression, as it is the polyphenolic molecule responsive to these activities.

2. MATERIALS AND METHODS

The inner barks of the species *Anacardium occidentale* L. were collected in the city of Canindé de São Francisco-SE. One specimen was registered under voucher ASE 40087 at the Herbarium of the Federal University of Sergipe (ASE).

Hydrochloric acid (HCl), acetic acid (CH₃COOH), boric acid (H₃BO₃), orthophosphhoric acid (H₃PO₄), methanol (CH₃OH), ethanol (C₂H₅OH), hexane (C₆H₁₄), sodium hydroxide (NaOH) and ethyl acetate (C₄H₈O₂) were purchased from Neon. (+) - catechin (C₁₅H₁₄O₆), 2,2 diphenyl-1-picryl hydrazil (C₁₈H₁₂N₅O₆) and gallic acid (C₇H₆O₅) were purchased from Sigma Aldrich.

2.1 Preparation and fractionation of hydroethanolic extract

To prepare the extract, the inner barks were dried in an oven at 37 °C with renewal and air circulation until complete dehydration. They were then powdered using a knife mill and a mass of 2.645 kg of the inner bark was subsequently subjected to extraction in 90% ethanol for five days by maceration. After this period, the extract was filtered and concentrated in rotary evaporator at

reduced pressure at 50 °C, yielding 96.9632 g and return of 10.63% of *A. occidentale* L. hydroethanolic extract. The obtained extract was stored under refrigeration.

Then, 70.00 g of HEE were dissolved in a CH₃OH/H₂O solution (2:3) and subjected to liquidliquid extraction with hexane and ethyl acetate. The following phases were obtained with the respective HEE yields: hexane phase - HP (2.75 g: 0.30%), ethyl acetate phase - EAP (7.61 g: 0.84%) and hydromethanolic phase - HMP (1.42 g: 0.15%).

2.2 Phytochemical prospecting

The plant's HEE and EAP were subjected to phytochemical prospecting according to the methodology described by Matos (2009) [16]. The methods used were only qualitative aiming to detect the occurrence of catechin, as well as other compounds in the HEE of *A. occidentale* inner barks in the phases.

2.3 Quantification of total phenolic compounds

The determination of the total content of phenolic compounds in the EAP of the *A. occidentale* L. inner barks was performed by spectrophotometry in the UV-Visible region, using the Folin-Ciocalteau reagent, following the methodology described by Galvão et al. (2018) [17]. All measurements were made in triplicate.

2.4 Quantitative evaluation of redox activity

Analysis of redox activity was realized by radical 2,2 diphenyl-1-picryl hydrazil (DPPH) sequestrant activity method, measurements was performed by spectrophotometry in the UV-Visible region. Consumption of DPPH free radicals for the samples was monitored throw the absorbances values decressement in solutions of differents concentrations [18].

This quantitative essay was developed using a UV-Vis spectrophotometer, model SP22, in a wavelength of 515 nm, with a positive control of gallic acid, in the range of 5, 10, 15, 20 and 25 μ g mL⁻¹.

2.5 Dpph calibration curve

For the calibration curve, 3 mg of DPPH were weighed and dissolved in 75 mL of methanol, obtaining a stock solution in the concentration of 40 μ g ml⁻¹. This solution was then cooled and protected from light. Dilutions in methanol were performed to obtain the final concentrations of 5, 10, 15, 20 and 25 μ g ml⁻¹. Calibration curve was developed by the absorbance values in 515 nm wavelength and all the measurements was made in a glass cuvettes with 1 cm optical path and methanol as blank, in triplicate with 1 min of interval between the scans. The equation obtained was C = 110.547 – 0.02804 x A, where C correspond to DPPH concentration, A is the absorbance value and correlation coefficient was R= 0.9983.

2.6 Absorbance measures

Absorbance measures of mixtures was performed in the 1°, 5°, 10° min and each 10 minutes until complete 60 min [17]. The mixture between methanol and sample was used as blank. From the calibration curve equation and absorbance values obtained in essay for all the concentrations analyzed was determined DPPH percentages remain, calculated, according to the equation:

where [DPPH] T corresponds to the concentration of DPPH in the medium, after the reaction with extract, and [DPPH] T0 to the initial concentration of DPPH.

The amount of antioxidant needed to decrease the initial DPPH concentration by 50% (IC₅₀) was calculated using the % DPPH_{REM} in 60 min. as opposed to sample concentrations. The results were

The absorbances at all concentrations tested at 60 min were converted into a percentage of inhibition (PI). The antioxidant activity was also expressed by the antioxidant activity index (AAI), calculated according to Hartati, Nadifan and Fidrianny (2020) [19], according to the equation:

$$AAI = \% DPPH_{REM} (FINAL) (\mu g mL^{-1}) / IC_{50} (\mu g mL^{-1}).$$

In this way, the AAI was calculated considering the mass of the samples tested in the reaction, thus resulting in a constant for each sample, regardless of the concentrations of DPPH and samples used. Antioxidant activity is considered unsatisfactory when the AAI value is less than 0.5, moderate when the AAI is between 0.5 and 1.0; strong when the AAI is between 1.0 and 2.0 and very strong when the value of the AAI is greater than 2.0.

2.7 High performance liquid chromatography (hplc) analysis

For this analysis, the Shimadzu liquid chromatograph Prominence series was used, consisting of two LC 6AD pumps, SIL 10 AF10 auto injector, DGU A5 degasser, solvent selector valve, SPD M20A photodiode array detector (PAD), connected to a CBM interface 20A and Rheodyne 7725 manual injector with 1 mL loop. For data acquisition and processing, the LC Solution software was used.

The EAP (1mg mL⁻¹) was filtered on nylon membranes of 2.5 cm x 0.45 cm and, first eluted (20 μ L) in a gradient system, with mobile phase water: Methanol 5-100%, for 60 min. with flow of 1 mL min⁻¹, in a 200-400 nm scan. Afterwards it was eluted again, however, under isocratic condition using the mobile phase 7% methanol for 10 min, with a flow of 1 mL min⁻¹, and detection in PAD-UV-Vis at 280 nm. The (+) - catechin standard was eluted under the same chromatographic conditions.

2.8 Preparation of support electrolyte solution

To perform voltammetric measurements, Britton-Robinson (B-R) buffer composed of orthophosphoric acid (5.05 mL), boric acid (4.64 g) and acetic acid (4.35 mL) was used as a support electrolyte. The pH adjustment was done by adding solutions of NaOH 2 mol L^{-1} and HCl 1 mol L^{-1} . The pH measurement was performed using the digital pHmeter model PG 1800.

2.9 Electrochemical study

The electrochemical techniques were performed in Autolab PGSTAT type II potentiostat in a system of three electrodes: vitreous carbon electrode (BAS, diameter 3.0 mm) as working electrode, platinum wire as auxiliary and Ag/AgCl (KCl 3 mol L⁻¹) as reference electrode. Data analysis was performed using graphics obtained in the experiments and treated with the aid of the Origin 8.0 software.

For the determination of redox activity by Cyclic Voltammetry (CV) and Differential Pulse Voltammetry (DPV) electrochemical tests were carried out in B-R buffer with EAP. Voltammetric measurements were performed in the potential range from - 0.2 V to + 1.4 V at a scan rate of 50 mV s⁻¹ to CV and 0 to 1.0 V at a scan rate of 10 mV s⁻¹ to DPV. These same analyzes were performed with the (+) - catechin standard. The study of the pH influence was performed using B-R buffer for pH values of 1.8, 3.4, 5.6, 7.0, 8.4, 9.4, 10.1 and 12.0.

3. RESULTS AND DISCUSSION

3.1 Phytochemical prospecting

Phytochemical triage of hydroethanolic extract and of ethyl acetate phase of *A. occidentale* inner barks detected different kinds of compounds, as steroids, phenols, flavones, flavonoids, saponins, tannins and xanthones, and are registred in Table 1.

 Table 1: Results for phytochemical prospection of hydroethanolic extract and of ethyl acetate phase of

 Anacardium occidentale inner barks. * + Presence; - Absence

Compounds	HEE	EAP
Anthocyanidins	-	-
Anthocyanins	-	-
Steroids	+	-
Fenois	+	+
Flavones	+	+
Flavonoids	+	+
Flavones	-	-
Leucoanthocyanidins	-	-
Saponins	+	+
Tannins	+	+
Triterpenoids	-	-
Xanthones	+	+

The classes of natural compounds with high antioxidant potential are phenolic acids, flavonoids and polyphenolic compounds. Presence of the reduced phenoxide radical resonates and stabilizes the free radical, which causes high antioxidant activity.

Anthocyanidins, anthocyanins, flavones, leucoanthocyanidins and triterpenoid were not detected. However, the negative results presented in this research, do not necessarily imply their absence, and it is likely that the amount of them is too small to be detected.

Factors such as seasonality, circadian rhythm, age or stage of development, temperature, water availability, UV radiation, mechanical stimulus and attack of pathogens, can influence the quantity and nature of the active constituents in the plant [20].

3.2 Quantification of total phenolic compounds

In this work, higher content of phenolic compounds was determined in EAP 848.62 \pm 78.18 µg mg⁻¹ of gallic acid/EAP. Another studies in etanolic extract samples of cashew peels, was observed contents around of 58.0 \pm 0.4 µg mg⁻¹ of gallic acid/HEE [9].

A study of the evaluation of *A. occidentale* extracts by the Folin-Ciocalteu method [9] showed that the inner barks are rich in phenolic compounds, with 345.16 ± 16.24 mg of gallic acid equivalents. Melo et al. (2008) [21] found values of total phenolics expressed in catechin equivalent in aqueous cashew extract, in the order of $808.05 \pm 32.32 \ \mu g \ mL^{-1}$. In contrast, the acetone extract had a content equal to $629.85 \pm 31.49 \ \mu g \ mL^{-1}$. In the work of Vieira et al. (2011) [22], the contents of total phenolic compounds found for the aqueous and hydroalcoholic extract of cashew, were 201.61 ± 19.15 mg of gallic acid in 100 g of pulp and 165.07 ± 4.10 mg of gallic acid in 100 g of pulp, respectively.

3.3 Quantitative evaluation of redox activity

The results obtained, expressed by the percentage of DPPH remaining of 5, 10, 15, 20 and 25 μ g mL⁻¹ of the EAP of *A. occidentale* in the time of 60 min, showed that with the increase of the concentration, there was also an increase in the DPPH radical sequestration. Thus, it was observed that the EAP of the plant's inner barks, rich in phenolic compounds, consumed more than 90% of the free radical DPPH.

The average effective concentration (EC₅₀) of the EAP was 16.134 μ g mL⁻¹, which corresponds to a strong antioxidant activity. The EC₅₀ data revealed that the EAP of *A. occidentale* inner barks has antioxidant activity and that it is related to the high content of phenolics.

However, the antioxidant activity index (AAI) may more accurately represent the antioxidant potential of a synthetic compound or natural product, against a free radical, than simply CE_{50} [21]. Thus, for the test performed, the value obtained for the AAI was 2.48 in the EAP of *A. occidentale*, reinforcing the results previously observed.

In Figure 1, the kinetic curves of decrease in the absorption of the DPPH radical can be seen when in the presence of EAP, in different concentrations. It is noticed that the EAP has a different behaviour according to the tested concentration. At a concentration of 5 μ g mL⁻¹, the low DPPH sequestration capacity was observed. However, it can be observed that the concentration that showed the greatest decay, that is, the greatest antioxidant capacity was 25 μ g mL⁻¹ in a shorter reaction time.



Figure 1: Kinetic curve of the antioxidant potential of Anacardium occidentale EAP by the DPPH method at different concentrations.

3.4 High performance liquid chromatography (hplc) analysis

Figure 2 shows the results of the EAP analysis of *A. occidentale* inner barks to confirm the presence of catechin by HPLC, through the evaluation of the major peaks of these compounds, in relation to the retention times and scanning spectra.



*Figure 2: Chromatographic EAP profile of Anacardium occidentale inner barks obtained by HPLC, in the condition of 5-100% exploratory water/methanol gradient, 280 nm, C*₁₈ analytical column, flow rate of 1 mL min⁻¹.

The analysis of the EAP chromatographic profile, in Figure 3, showed a behavior similar to that of the catechin standard in Figure 4. The EAP had its compounds eluted in a single package between the time of 3 to 5 min, presenting a little broad base and the presence of a major peak eluted in 3 to 4 min. In the analysis of the two chromatographic profiles, EAP and CAT, can notice a characteristic common to them: the presence of a peak, in the time interval of 3-4 min.



Figure 3: Chromatogram and UV-Visible spectrum of EAP, subjected to isocratic elution, mobile phase water: methanol 7%, detection in PAD-UV-Vis at 280 nm, injection volume of 20 μ L, flow rate of 1 mL min⁻¹, for 10 min, analytical column C₁₈.

The absorption spectrum in the region of UV-Visible corresponding to EAP, as well as the corresponding spectra of the catechin standard are shown in Figures 3 and 4, respectively.



Figure 4: Chromatogram and UV-Visible spectrum of the (+) - catechin standard, subjected to isocratic elution, mobile phase water: 7% methanol, detection in PAD-UV-Vis at 280 nm, injection volume of 20 μ L, flow rate of 1 mL min⁻¹, for 10 min, analytical column C₁₈.

The data obtained show that the absorption band of the EAP UV-Visible spectrum of *A. occidentale* L. had its maximum absorption at 274 nm, which resembles the standard tested catechin, which was obtained at 278 nm, corroborating with the results obtained in works present in the literature [23, 24].

3.5 Eletrochemical study

Voltammetric studies for (+) - catechin standard solution showed two anodic signals around of 0.31 and 0.65 V, respectively, and one cathodic signal in 0.23 V, to cyclic voltammetry analysis as shown in Figure 5.



Figure 5: Cyclic Voltammetry voltammograms to $4.27 \ 10^{-3} \text{ mol } L^{-1} \text{ standard } (+)$ - catechin solution in B-R buffer pH 5.6 with 500 mV s⁻¹ of scan rate.

Differential pulse voltammetry showed two anodic peaks in 0.24 and 0.63 V as shown in Figure 6.



Figure 6: Differential Pulse Voltammetry voltammogram to $4.27 \ 10^{-3} \ mol \ L^{-1}$ standard (+) - catechin solution in B-R buffer pH 5.6 with 10 mV s⁻¹ of scan rate.

According to Janeiro and Brett (2004) [25], the first oxidation that occurs in ring B of the Catechin refers to a reversible reaction, which differs from the second oxidation that does not have such potential for radical scavening, such a reaction is shown in Figure 7.



Figure 7: Schematic representation of the oxidation mechanism of (+) - catechin. Extracted from [25].

The analysis of the cyclic voltammetry of the EAP (Figure 8) showed two anode signals around 0.34 and 0.68 V, respectively, and revealed the non-occurrence of the reduction peak. This result may be related to the reaction of species formed during oxidation, with the other components of the EAP, which makes the oxidation reaction totally irreversible, different from the analysis of the CAT standard.



Figure 8: Cyclic Voltammetry voltammograms to 4.27 10^{-3} mol L⁻¹ of EAP in B-R buffer pH 5.6 with 500 mV s⁻¹ of scan rate.

The differential pulse voltamogram for the EAP (Figure 9) shows the overlapping of signals for the first oxidation peak, which may be linked to the oxidation of the other polyphenols compounds present in the EAP of *Anacardium occidentale*.



Figure 9: Differential Pulse Voltammetry voltammogram to 4.27 10⁻³ mol L⁻¹ of EAP in B-R buffer pH 5.6 with 10 mV s⁻¹ of scan rate.

In the study of the influence of pH variation on the redox potential of *A. occidentale*, analyses were performed on 8 different pH values, varying between 1.8 and 12.0 and keeping the EAP concentration constant $(4.27 \ 10^{-4} \ mol \ L^{-1})$, results are showed in Figure 10.



Figure 10: Differential Pulse Voltammetry of EAP of Anacardium occidentale in 4.27 10⁻⁴ mol L⁻¹ showing the variation of anodic peak current and potential in function of the pH, in B-R buffer with 10 mV s⁻¹ of scan rate.

The graph expressed in Figure 11 shows the linear variation of the oxidation potential in relation to the pH for peak 1 and peak 2, with the least and most positive potential, respectively. The potential values for pH 12.0 could not be added, as there are no appreciable oxidation peaks for the concentration evaluated at this pH.



Figure 11: Variation of oxidation potential in relation to pH of EAP of Anacardium occidentale in 4.27 10^{-4} mol L^{-1} .

The analysis of the results obtained shows the decrease in the current intensities of the anodic peaks in relation to the increase in the pH values. This effect suggests that the use of a more acidic medium provides the protonation of the species of interest, which facilitates its subsequent oxidation and, consequently, the increase in analytical signals.

It is also possible to notice the displacement of the potential for occurrences of oxidation peaks to less positive values as the pH increases, suggesting lower energy expenditure in the electronic transfer process, despite incurring a loss of current intensity.

A study of the effect of the analyse adsorption on the glassy carbon electrode surface in successive scans without the surface renovation was carried out. The differential pulse voltamograms shown in Figure 12 show the reduction in current intensities for the anode peaks of the EAP when the sensor surface is not renewed between analyses.



Figure 12: Differential Pulse Voltammetry voltammograms to successive scans without renewing the surface 4.27 10⁻³ mol L⁻¹ of EAP in B-R buffer pH 1.8 with 10 mV s⁻¹ of scan rate.

The components of the EAP in addition to the products generated during oxidation are strongly adsorbed on the electrode surface, which responds by decreasing the intensity of the analytical signals obtained in the measurements.

4. CONCLUSIONS

Through the phytochemical screening of the hydroethanolic extract and the ethyl acetate phase of *Anacardium occidentale* it was possible to identify the presence of some classes of secondary metabolites such as: organic acids, reducing sugars and phenols, which are of pharmacological interest, thus allowing the discussion between scientific knowledge and the popular use.

With the results obtained from the quantification of EAP total phenolics, it was possible to verify the large amount of these compounds, present in the inner bark, and the redox activity became even more evident, after the evaluation of DPPH free radical sequestration, consuming more than 90% of the free radical DPPH. Thus showing a very strong antioxidant activity.

HPLC analysis of the crude extract in EAP of the inner barks of *A. occidentale*, the presence of catechin was observed, through the assessment of major peaks, retention time and scanning spectrum of these compounds, it helps to identify the participation of catechin in the redox activity.

The determination of the redox profile of the crude extract in EAP of the inner barks of *A*. *occidentale* L. using voltammetric techniques CV and DPV, showed similarity with that of CAT, reaffirming the relationship between the antioxidant activity and the presence of catechin.

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