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Short Communication

Electrochemical Behavior and Antioxidant Activity of Hibalactone

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*Hydrocotyle umb*ellata L. is a groundcover plant that has been used in the folk medicine of Argentina, Brazil, Cuba e India, mainly to reduce inflammatory processes. The lignan, hibalactone, can be isolated from the roots of the plant. In the anti-inflammatory and antioxidant activity of such natural products, it is believed that the mechanism of action involves the butyrolactone ring, which would act as free pharmacophore. In this study, electrochemical, spectrometric and computational chemistry approaches were used to evaluate the lignan antioxidant activity and electro-oxidation pathway. Electrochemical measurements were performed in a 3 mL cell, operated in a three electrode system, consisting in an Ag/AgCl reference electrode, platinum as counter electrode and glassy carbon electrode was used as working electrode. Radical scavenging assays were performed with DPPH and ABTS. In the computational chemistry approach geometry, optimization was performed with semi-empirical calculations (PM3) and the orbitals/charges calculations were performed by the extended Hückel method. The voltammetry showed a very slight variation with pH alteration, which can be justified by lactone hydrolysis that occurs in acidic and basic pH. In the HOMO and total charge density distribution it was possible to observe that the higher prevalence was in the benzo[1,3]dioxole rings and in the alkenes/alkanes in proximity with the lactone ring. The hibalactone displayed a single quasi-reversible electro-oxidation. Given the HOMO and charge density distribution in the lignan, more so the single oxidation in the evaluated potential, it is reasonable to infer that the alkene bonded with the lactone ring was a probable site of oxidation.

Keywords: electrochemical index, voltammetric profile, redox behavior, natural antioxidants

1. INTRODUCTION

Medicinal plants represents an important source of income for underdevelopment countries and a promising therapeutic alternative for the population. Hence, the knowledge about folk plants and

their components is one of the most efficient strategies to triage the vast biodiversity, as well as, to drive the scientific research about the chemistry and pharmacology of natural products [1].

The Araliaceae family from Apiales order is divided in around 40 genus, in which more than 1500 species have already been identified, whereas almost 50 species from the genus *Aralia, Dendropanax, Hydrocotyle, Oreopanax, Pentapanax e Schefflera* belongs to this family, and are commonly found in Brazil [2].

The genus *Hydrocotyle* L. (Araliaceae) embrace more than 130 species spread over tropical and temperate regions of the world, mostly typical of aquatic or wet habitats [3]. The phytochemical screening of the *Hydrocotyle* genus revealed a great diversity of bioactive chemicals of variable structure, including flavonoids, flavones, saponins, triterpenes and other phenols [4].

The *Hydrocotyle umb*ellata L., is a groundcover plant, typical of resting biome, popular known as "acaricaba", "acariroba", "acariçoba", "barbarosa", "para-sol" and "erva-do-capitão" [5]. The decoction of *H. umbellata* leaves has been used in the folk medicine of Argentina, Brazil, Cuba and India, mainly in topical treatment of dermatitis and also oral to reduce inflammatory processes [6]. The anti-inflammatory activity was also observed for ethanolic extracts of *H. umbellata* roots, which showed also anti nociceptive response in rats [7].

The major secundary metabolites identified in both, leaves and roots, were triterpenes, saponins, flavonoids, poliacetylenic and leuco ceramides compounds [5].

The purification of *H. umbellata* leaves extracts resulted in the isolation and elucidation of new phytochemicals [8]. In this work, the lignan hibalactone, (IUPAC name = (3E)-4-(1,3-Benzodioxol-5-ylmethyl)-3-(1,3-benzodioxol-5ylmethylene)dihydro-2(3H)-furanone)), was isolated for the first time from ethanolic extract of *H. umbellata* underground parts (Fig. 1).

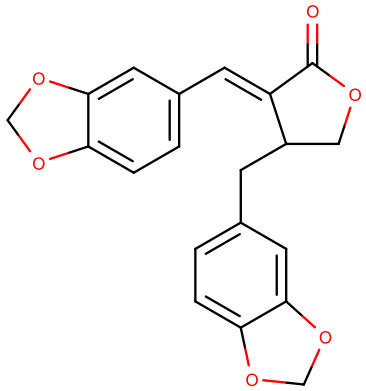

Figure 1. Chemical structure of hibalactone.

This dibenzylbutirolactone was also isolated from other species *i.e. Anthricus sylvestris* [9], *Zanthoxylum naranjillo* [10], *Micranthemum umbrosum* [11], *Chamaecyparis pisifera* [12], *Artemisia chamaemelifolia* [13], *Juniperus chinensis* [14] and *Justicia neesi*, all presenting rich proportions of lignans and recognized biological activities [15].

The biological properties of lignins and lignans, including anti-inflammatory and antioxidant activity has urged in the development of new therapeutic compounds [16-20].

Some anti-inflamatory mechanisms of action can be related to the capacity to prevent oxidative processes [21]. Taking into account, that the antioxidant and many other biological properties of

natural products have strong connection with their redox behavior, the electrochemical characterization may offer new insights. Moreover, despite the pharmacological relevance of hibalactone, to the best of our knowledge there no study about the electrochemical behavior of this natural compound.

Henceforth, this paper aims to provide new qualitative information and also quantitative alternatives for hibalactone by means of voltammetric approaches.

2. MATERIALS AND METHODS

2.1. Reagents and Solutions

$ABTS^{\cdot+}$ (2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)), DPPH (1,1-diphenyl-2-picrylhydrazyl) and the other reagents used in analyses were of analytical purity and were obtained in Sigma Chemical Co. (St. Louis, MO, USA). All electrolytic solutions were prepared with purified water in the Millipore Milli-Q system (conductivity $\leq$ 0.1µScm $^{-1}$) (Millipore S. A., Molsheim, France). Alumina Nº3 from Arotec (Cotia - SP) and ethanol and methanol were obtained from Panreac AppliChem. The hibalactone was isolated from crude extracts of *H. umbellata* subterraneous parts with dichloromethane liquid-liquid partition.

2.2. Electrochemical analyses

Electrochemical measurements were performed in a potentiostat/galvanostat µAutolab III, integrated with *software* GPES 4.9 (Eco-Chemie, Utrecht, The Netherlands). In the construction of the graphs and to perform statistical analyses, the program Origin 8.0 was used. Analyses were performed in an electrochemical cell with 3 mL volume, operated in a three electrode system, consisting in a reference electrode Ag/AgCl (KCl 3 mol.L^{-1}), platinum electrode as counter electrode and glassy carbon electrode (1.0 mm²) was used as working electrode. Before each analysis, glassy carbon electrode was polished using Arotec Alumina nº3 and, posteriorly, the electrode was placed in a phosphate buffer solution (PBS) pH 9.0 and electrochemically cleansed with cyclic voltammetry, performed 0 at 1.0 V and scan range from 0 to 1.4 V. Then, the electrode was washed with deionized water. The cleaning proceedings were performed until a stable base line was obtained.

Cyclic voltammetry (CV) assays were performed with scan rate of 100 mV.s^{-1} and potential range from 0 to 1.4 V. 1.0 mL PBS 0.1 M pH 6.0, 0.9 mL methanol and 0.1 mL from the 1 mg/mL analytical solution (in methanol) were transferred to the electrochemical cell.

Differential pulse voltammetry (DPV) was performed with 50 mV pulse amplitude and scan rate of 10 mV.s^{-1}. 1.0 mL PBS 0.1 M, 0.9 mL methanol and 0.1 mL from the 1 mg/mL analytical solution (in methanol) were transferred to the electrochemical cell, in the assays. This analysis was performed in different pHs 0.1 M PBSs (4, 5, 6, 7, 9) and, then, different analytical concentrations (12.5, 25, 37.5, 50, 75 µg/mL), 0.9 mL methanol and 1.0 mL 0.1 M pH 6.0 PBS, 50 mV pulse amplitude and 10 mV.s^{-1}. All experiments were performed in environmental temperature (21 ± 2 ºC) in duplicate (n = 2).

Square wave voltammetry (SWV) was performed with 50 mV pulse amplitude and 30 Hz pulse frequency and 100mV.s^{-1} scan rate. 1.0 mL PBS 0.1 M, 0.9 mL methanol and 0.1 mL from the 1 mg/mL analytical solution (in methanol) were transferred to the electrochemical cell.

2.3. DPPH and ABTS radical scavenging assays

The DPPH assay was performed according to Brand-Williams [22] and Arteaga [23] with modifications. Briefly, ca. 0.5 mL of ethanol was added to 2.5 mL of DPPH (0.1 mM) in order to reach A = 0.7, λ = 517 nm and a final volume of ca. 3.0 mL that was repeated for the ethanolic samples of hibalactone (1 mg/mL) and gallic or ascorbic acid (1 mM). The reaction solution was incubated for 5 to 15 min in the dark at room temperature and measured with UV-Vis spectrophotometer (Jasco® V-530) at 517 nm, against the blank (A ~0.7), whereas ethanol, the solvent used to prepare all solutions, was used in order to adjust the baseline (A = 0.000). Antioxidant activity was expressed as EC50, representing the amount of analytical sample to produce 50% of decolorization of DPPH• relative to the blank control.

The ABTS (2, 2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)) was performed according to Re et al [24]. The radical ABTS•+solution was generated by reacting 5.0 mL of 7 mM ABTS with 88 µL of 140 mM Potassium persulfate in the dark at room temperature for 16 hours. Then, the radical solution was diluted with ethanol to reach an absorbance of 0.7 (λ = 734 nm). The 100 to 500 µL of analytical samples (hibalacotone and antioxidant standard) were added to this final radical solution, always respecting the same final volume. The reaction solution was incubated at dark and room temperature for 5 to 15 minutes and the percentage of decolorization was converted in means of gallic acid or ascorbic acid equivalents.

2.4. Computational chemistry calculations

Geometry optimization was performed with semi-empirical calculations (PM3) and the HOMO (Highest Occupied Molecular Orbital) orbitals/charges calculations were performed by the extended Hückel method. The GAMESS 09 software with MoCalc2012 interface were used to perform the calculations. Chem3D Pro 12.0 was used to render the graphical images.

2.5. Statistical analysis

The statistical data of RSD (Relative Standard Deviation), average of triplicates, SD (Standard Deviation) and r^2 were calculated in the software Origin 8.0.

3. RESULTS AND DISCUSSION

The electrochemistry behavior of hibalactone was investigated by means of voltammetric methods. The CV (Figure 2) showed an irreversible anodic peak, 1a, at E_{pa} ~ 1.0 V, on the reverse scan

no cathodic peak was observed, whereas at successive scans a prominent decrease of peak currents occurs. This redox behavior is in agreement with the redox reactions leading to the formation of insulating film at electrode surface.

The redox irreversibility was ascertained by SWV experiments (Figure 3).

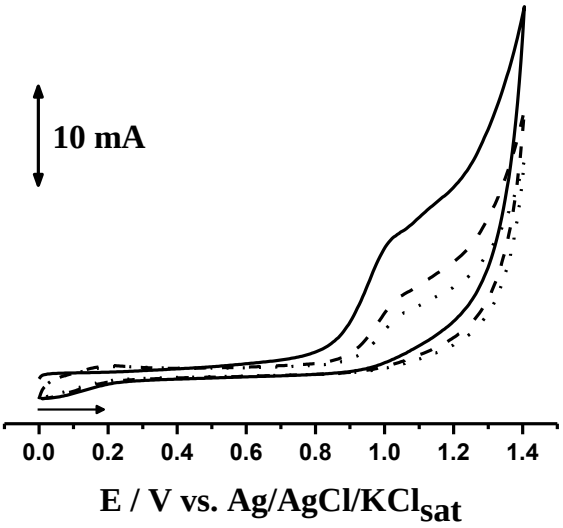

Figure 2. Successive cyclic voltammetry scans obtained for 50 µg/mL hibalactone isolated from *H. umbellata* at GCE in 0.1 M PBS, pH 6.0, 1ª scan (—), 2ª scan (--) e 3ª scan (···).

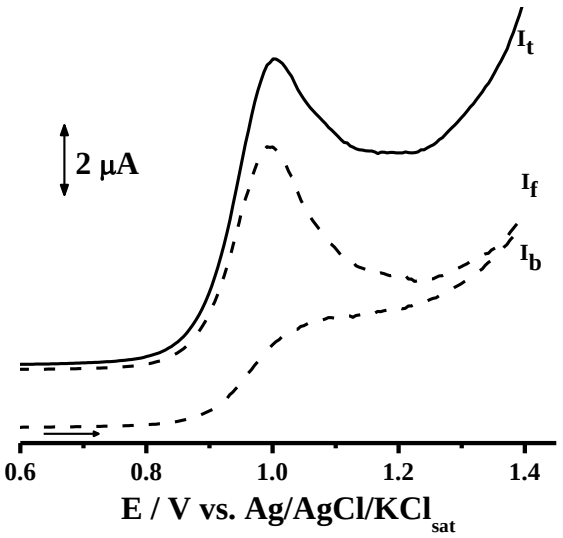

Figure 3. Square wave voltammetry calculated for hibalactone isolated from *H. umbellata* at CGE in 0.1 M PBS pH 6.0 (I_t = Total current; I_f = Forward current; I_b = Backward current). Pulse frequency = 30 Hz, scan rate = 100mV.s^{-1}, pulse amplitude = 50 mV.

The irreversibility and high positive potential value is not consistent with natural products with high antioxidant activity. In fact, the benzodioxole (1,2-methylenedioxybenzene) structure of hibalactone does not present functional groups with recognized reducing power, *i.e.* o-di-phenols. From the scarce literature about the electrochemistry of lignans, it was found that the electrochemical

oxidation of phenolic derivatives isolated from hexane extract of *Iryanthera juruensis* (Myristicaceae) fruits in aprotic medium occurred at E_{pa} ca. 0.8 V, whereas for the ones presenting similar 1,2-methylenedioxy group, the anodic process occurred at 1.5 V [25].

In fact, the antioxidant capacity of hibalactone, was also performed by DPPH and ABTS spectrometric assays. The radical scavenging activity of lignan was compared with ascorbic acid and gallic acid. The results obtained for hibalactone was far lower than the one observed for such usual antioxidant standards. The results are presented in Table 1.

Table 1. DPPH and ABTS radical scavenging performance of hibalactone, ascorbic acid and gallic acid standards.

Spectrometric assay	EC_{50} Ascorbic acid (µg/mL)	EC_{50} Gallic acid (µg/mL)	EC_{50} Hibalactone (µg/mL)
DPPH	2.532 ±0.215	0.9725 ± 0.124	392.471 ± 2.973
ABTS	2.04	0,474	309.54 ± 76.980

*EC_{50} results are shown as average±SD (assays were performed in triplicate, n = 3)

Similar results were observed with the lignans, sesamin and sesamolin, that exhibited lower radical scavenging ability than sesamol due the absence of free hydroxyl group in its structure [26].

In order to check the participation of proton on the electrochemical oxidation of hibalactone, the DPV were performed in different pHs (Figure 4).

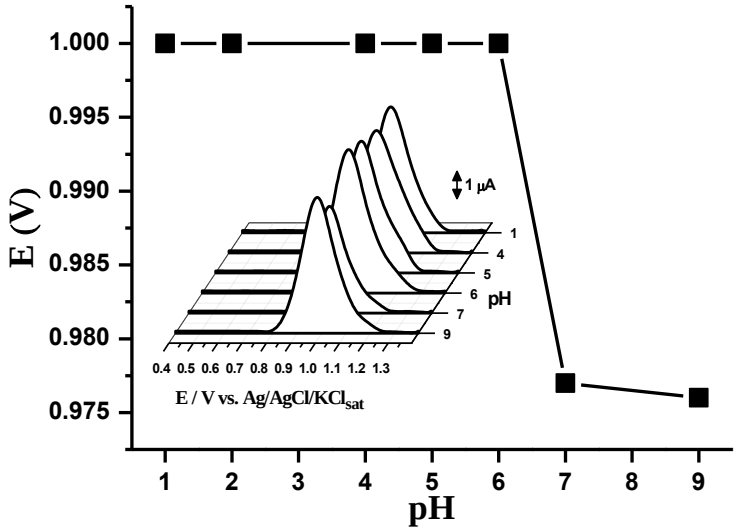

Figure 4. Plot of peak potential *versus* PBS pH value. Inset: Differential pulse voltammograms of hibalactone isolated from *H. umbellata* in different pH values of 0.1 M PBS. Pulse amplitude = 50 mV and Scan rate = 10 $mV.s^{-1}$.

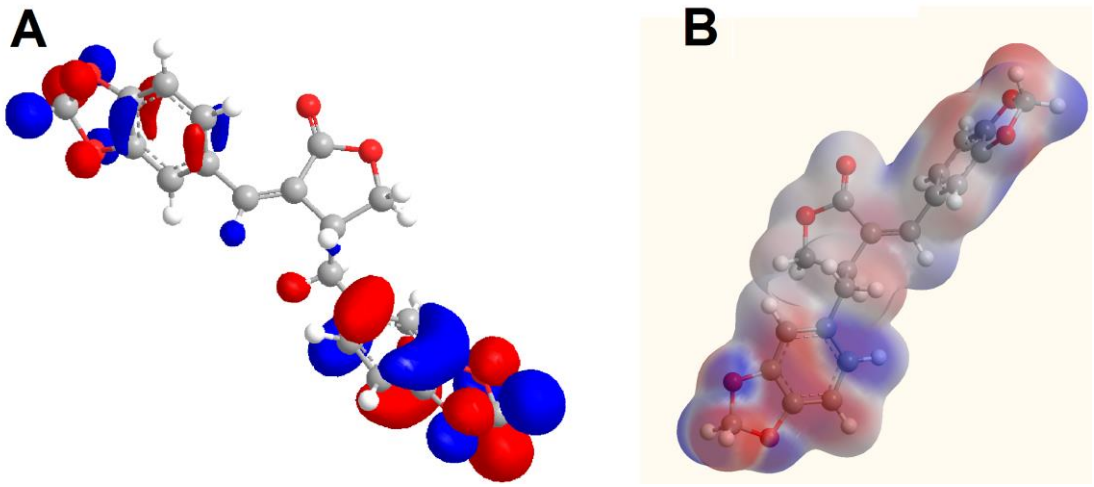

Figure 5. Hibalactone HOMO orbital distribution (A) and total charge density (B) for the lowest energy conformation.

It can be noticed in figure 4, that from pH 3 to 9, no peak shifting is observed, which is non typical behavior for redox reactions involving organic compounds. Indeed, it should be expected a decreasing of anodic potential, as the pH increased. Moreover, the slope, *E*p *vs* pH, for redox reactions involving the transference of equal number of electrons and protons may present the theoretical "Nerstian" value of 59 mV/pH. The very slight can be justified by non-electrochemical reactions, which is consistent with the presence of three very unstable groups, namely one lactone and two dioxole rings. The prior chemical cleavage of methylenedioxy group in an acid catalyzed process was previously attributed to the electrochemical oxidation of sesaminol at ca. 1.2 V [27].

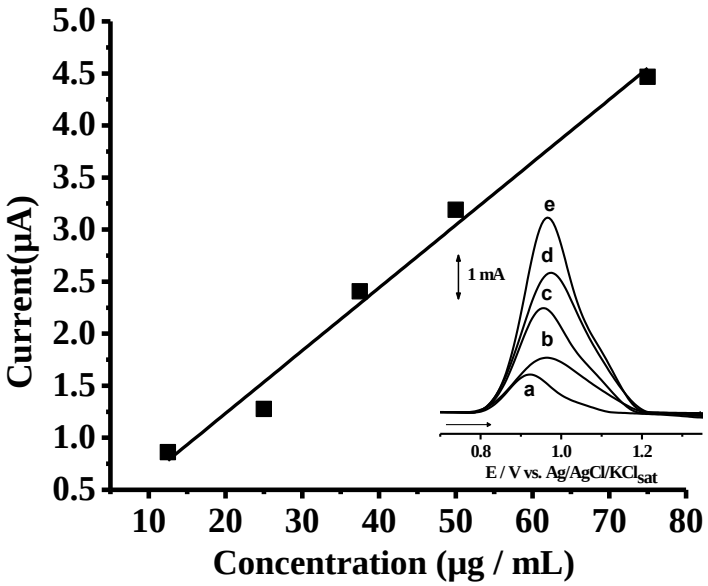

Figure 6. Calibration curve obtained from DPV assay with increasing concentrations of hibalactone (**a**: 12.5; **b**: 25; **c**: 37.5; **d**: 50 and **e**: 75 µg/mL). Inset. The related DP voltammograms.

The semi-empiric HOMO and total charge density distribution calculations for hibalactone, were made for the lowest energy conformation (Figure 5).

It was possible to observe that the higher prevalence was in the benzo[1,3]dioxole rings and in the alkenes and alkanes in proximity with the lactone ring. Hence, it can be inferred that electrochemical oxidation process may involve an initial attack at these groups, followed by the formation of unstable radicals and polymeric chain reactions.

Despite the strong adsorptive behavior, the DPV at glassy carbon electrode (GCE) was also evaluated for quantitative purposes. This assay was performed at increasing concentrations of hibalactone in PBS, pH 6 (Figure 6). A linear calibration curve was obtained (r = 0.99). The linear regression equation calculated was y = 0.06028.x + 0.02903. Moreover, to evaluate repeatability, multiple assays where performed in the same day (n = 3) and in different days (n=3, from 3 to 3 days). RSD value for intra-day analysis was (±5.5%) and for inter-day analysis was (±7.6%).

It was possible to observe a linear behavior between peak currents and hibalactone concentration, allowing the use of DPV for quantitative determinations of this and other lignans.

4. CONCLUSION

The hibalactone exhibited a single irreversible electro-oxidation at positive potential of 1.0 V, which may involve EC mechanisms followed by polymerization reactions. The HOMO and charge density distribution in the hibalactone core, allow to infer that the benzo[1,3]dioxole, followed by the alkene site nearby lactone ring must be the most vulnerable sites of electrochemical oxidation.

From DPV assays performed with increasing concentrations of hibalactone, as well as, under systematic polishing procedures, a calibration curve was obtained with satisfactory coefficient of correlation and repeatability, allowing quantitative determinations.

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