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**Calligonum polygonoides** Linnaeus Extract: HPLC-EC and Total Antioxidant Capacity Evaluation

Sara M. C. Gomes,[a] Isabel P. G. Fernandes,[a] Narpat Singh Shekhawat,[b] Sunita Kumbhat,[c] and Ana Maria Oliveira-Brett*[a]

**Abstract**: Flavonoids in *Calligonum polygonoides* Linnaeus extract were separated, detected, and identified by reverse-phase high-performance liquid chromatography (RP-HPLC) with electrochemical detection (EC) in combined isocratic and gradient elution using a glassy carbon or a boron doped diamond electrode. Ultrasonication coupled with a microwave-assisted technique was developed to optimize the extraction of the phenolic compounds. The total antioxidant capacity was quantified using the DPPH method and voltammetry. The RP-HPLC-EC led to the detection of nine different flavonoids: catechin, delphinidin, fisetin, myricetin, epicatechin, kuromanin, rutin, callistephin and procyanidin A2, in a single run by direct injection of the sample extract solution.

**Keywords**: *Calligonum polygonoides* Linnaeus · DPPH method · Electrochemical detection · Flavonoids · Reverse-phase high-performance liquid chromatography

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**1 Introduction**

Phenolic compounds constitute one of the most numerous groups of plant secondary metabolites. The interest in the bioactivity of phenolic compounds is due to the potential health benefits of these polyphenolic components and their involvement in important biological and industrial processes. Flavonoids can be found in fruits, vegetables, tea, wine and chocolate. The basic structure of flavonoids consists on the tricyclic **C₆/C₁/C₆**. Their benefits to people are anti-inflammatory, hormonal, anti-bleeding, anti-allergic and even prevention of the cancer onset. It is also attributed to flavonoids the improvement in hair and nails condition since they promote the vitamin C absorption. However, the most important is their antioxidant property, which fights free radicals in the human body, detoxifying the body, and making better use of nutrients. The consumption of fruits and vegetables has long been associated with health benefits due to their high antioxidants concentration.

The genus *Calligonum* (family *polygonaceae*) comprises about 80 species, has numerous therapeutic benefits already known and studied, and are distributed throughout northern Africa, southern Europe and western Asia. *Calligonum polygonoides* Linnaeus, locally known as Phog or Phogra, is a common woody shrub grown naturally in sand dunes of the desert of Rajasthan, in India [1] and it is well known for its adaptability in extreme xeric conditions. The plant is ecologically valuable as it stabilizes the sand dunes and prevents soil erosion, and is also an important source of food [1,2]. It has leafless fleshy green smooth branches and white stem and produce small numerous flower buds and succulent flowers that are converted in hairy/spiny fruits. The over exploitation of the plant has made it vulnerable and endangered species in its habitat [2].

*Calligonum polygonoides* Linnaeus is known for its medicinal properties apart from being traditionally used as food during frequent famines, Figure 1 [3–6]. These plants produce numerous flower buds during March and April. The local people harvest branches bearing on opened but still green flower buds, sun-dry, harvest and store them, as valuable condiment/vegetable, “phog” flowers or “phogla”. The vegetable “phogla” is used to make delicious heat relieving “Raiyta” (a curd/yogurt preparation) by locals. The flowers of the plants are known to contain a high amount of protein, possess tonic and digestive properties, being also useful against asthma, cough and cold. The juice is applied to the eye as an antidote to scorpion sting, the latex is used to treat eczema, bite dogs and abortion, and the roots decoction mixed with catechu is used as gargle for sore gum. Phytochemical screening of *Calligonum polygonoides* shows the presence of flavonoids, alkaloids, proteins, tannins, steroids, phenols, carbohydrates and terpenoids in flower buds, flowers, seeds, and stems [7–11].

Thus, owing to the health properties associated with these shrubs, in which the phenolic antioxidants play...
a crucial role, it is indispensable to have reliable methods capable of measuring their antioxidant activity.

Many different methods can be used to measure the total antioxidant activity, the DPPH \(^{C}\) (1,1-diphenyl-2-picrylhydrazine) method measures the radical scavenging activity [12–15], presenting advantages when compared to other spectrophotometric methods such as ABTS(2,2’-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) method [16–18], because the DPPH \(^{C}\) does not require the generation of the radical. In fact, the DPPH \(^{C}\) is a commercial radical that can be reduced by antioxidant molecules, changing its color from purple to yellow after the reaction, and causing the absorbance decrease at the wavelength \(\lambda \approx 516\) nm.

The electrochemical techniques, mostly voltammetric techniques, are applied to the study of electroactive species, including the antioxidant compounds, in order to evaluate the antioxidant activity of plant extracts, herbal products or other compounds [19–22] presenting many advantages such as speed of analysis, low cost, simplicity and low consumption of reagents when compared to other methods [23]. It’s important to note that the voltammetric techniques depend only on the inherent electrochemical properties of antioxidants present in the sample.

Antioxidants can act as reduction agents and tend to be easily oxidized at inert electrodes, enabling the establishment of a relationship between electrochemical behavior and “antioxidant power”, the lower the oxidation potential the greater the antioxidant power [24].

The presence of voltammetric signals at low anodic potentials indicates the presence of polyphenolics of high antioxidant capacity, whereas the polyphenolics with low antioxidant activity show electrochemical activity at higher oxidation potentials [25].

The electrochemical behavior of flavonoids, such as catechin (flavan-3-ols) and epicatechin (flavonols), that exhibit an identical oxidation potential [24,26], are very good antioxidant electrochemical standards.

The electrochemical index \((EI)\) was defined as the total phenolics concentration obtained using electrochemical techniques for predominant and representative phenolic classes, taking into account the peak potential \((E_{ap})\) and peak current \((I_{ap})\) [23,24,26].

The lower peak potential corresponds to greater electron donor ability and higher peak current, and the \(EI\) is calculated using the equation [23]:

\[
EI = \left( \frac{I_{ap1}}{E_{ap1}} \right) + \left( \frac{I_{ap2}}{E_{ap2}} \right) + \cdots + \left( \frac{I_{apn}}{E_{apn}} \right)
\]

Extraction of phytochemicals from plant materials is the most important step before further analysis, and it has been accomplished by traditional extraction processes, such as solid-liquid extraction, using solvents such as methanol, ethanol and acetone, and also through steam distillation. The separation process consists in the distribution of analyte between two immiscible phases. It is important that the extraction processes occurs with selective separation of the target components from the sample at maximum amount and/or interferences elimination.
One of the simplest extraction techniques is the ultrasonic extraction that is easy to perform in an ultrasonic bath. In this method, the crushed sample is mixed with a suitable solvent and placed into the ultrasonic bath where the working temperature and extraction time are set [27–29]. Extraction of the flavonoids from seeds has been performed by ultrasonication, using hydrochloric acid in methanol as extraction solvent (MeOH/HCl (99:1) v/v) [28,30]. In order to increase the efficiency of the extraction procedure, microwave radiation has also been used before ultrasonication [31].

Reverse-phase high-performance liquid chromatography (RP-HPLC) methods with electrochemical (EC) glassy carbon detectors have been widely used for the separation, detection and identification of polyphenols in different extracts and plant materials [30,32,33]. The electrochemical detectors enable highly selective and sensitive results, and it is possible to modify the experimental conditions, such as the electrolyte ionic strength, buffer and pH, in order to maximize the sensitivity.

The boron doped diamond electrode (BDDE) electrochemical properties are different from other electrode materials (glassy carbon, pyrolytic graphite and carbon paste): a wide working potential window in aqueous solutions, low and stable background current, long term stability and low sensitivity to dissolved oxygen, good response for several redox analytes without any conventional pretreatment, weak adsorption of polar molecules and optical transparency in the UV/Vis and IR regions of the electromagnetic spectrum, useful properties for spectroelectrochemical measurements [34].

Polarographic and spectrophotometric assays and HPLC with UV detection, since polyphenols absorb UV and visible light, have also been used [27,35].

The aim of this study was the separation, detection, and identification of the phenolic compounds of flower buds/immature flowers of \textit{Calligonum polygonoides} Linnaeus (CP)., using for the first time RP-HPLC-EC with the detectors: glassy carbon electrode or a boron doped diamond electrode, and the quantification of CP total antioxidant capacity by differential pulse voltammetry, using a glassy carbon electrode, and by the DPPH method.

2 Experimental

2.1 Materials and Reagents

The \textit{Calligonum polygonoides} Linnaeus (CP) solid and dry plant material, consisting predominantly of dried immature flower buds, from sand dunes of the desert of Rajasthan, was provided by the Department of Botany, Jai Narain Vyas (J.N.V.) University, Jodhpur, India.

Cyanidin-3-O-glucoside chloride (kuromanin chloride), myricetin, fisetin, procyanidin A2, delphinidin chloride, (-)-epicatechin, callistephin chloride, malvin chloride, cyanin chloride, isorhoifolin and morin hydrate were from Extrasynthese, Genay, France. Catechin hydrate, rutin hydrate, quercetin dihydrate and chrysin, were from Sigma-Aldrich, Madrid, Spain. DPPH free radical form was obtained from Sigma-Aldrich, Madrid, Spain. Methanol HPLC-grade and formic acid were from Merck, Darmstadt, Germany.

All solutions were prepared using analytical grade reagents and purified water from a Millipore Milli-Q system (conductivity $\leq 0.1$ $\mu$S cm$^{-1}$).

Stock standard solutions of $\sim10$ mM of the phenolic compounds were prepared in the HPLC mobile phase, 83$\%$ purified water, 16$\%$ methanol and 1$\%$ formic acid. The stock solution flasks were protected from light with aluminum foil, kept in a refrigerator at 4°C, and re- mained stable for at least one month. These solutions were appropriately diluted to $10^{-4}$M and from this concentration the standard solutions for direct injection into the HPLC system were prepared. Working solutions were freshly prepared when needed.

The supporting electrolytes, pH 1.9 (0.1 M HCl + KCl) and pH 6.9 (0.1 M phosphate buffer NaH$_2$PO$_4$ + Na$_2$HPO$_4$), were prepared using analytical grade reagents and purified water.

2.2 Equipment

2.2.1 HPLC

HPLC analyses were performed using a Waters 2690 liquid chromatograph, with a Waters 996 photodiode array detector (PDA) and a Concorde electrochemical detector (EC) from Waters, Milford, MA, USA. Data acquisition and remote control of the system were done by Empower Workstation Chromatography Manager from Waters Corporation. Chromatography separation of phenolic compounds was carried out using a 150 mm × 4.6 mm i.d., 5$\mu$m reversed-phase (RP) Inertsil ODS-C18 steel column with a guard column of the same type from GL Sciences Inc., Tokyo, Japan.

The electrochemical detector was a VT-03 flow cell from Antec Leyden, Zoetewoude, Netherlands, of a confined wall-jet cell design, in a three-electrode configuration, a GCE (d=2 mm), an in situ Ag/AgCl reference electrode and a stainless steel auxiliary electrode. The in situ Ag/AgCl reference electrode, referred to here as ISAAC (in situ Ag/AgCl) is in direct contact with the mobile phase that contains 2 mM chloride ions (2 mM KCl). There is a difference of +0.2 V between the potential of the Ag/AgCl (saturated KCl) reference electrode and the ISAAC reference electrode in contact with 2 mM KCl. Thus, for an experiment running at +0.7 V vs. Ag/AgCl with saturated KCl, the potential setting using ISAAC should be +0.51 V.

All measurements were performed at the applied cell potential of $E_{ap}=+0.5$ V vs. ISAAC ($E_{ap}=+0.69$ V vs. Ag/AgCl (saturated KCl) reference electrode).

A thin-layer cell design electrochemical flow detector with a BDDE (d=6 mm) working electrode and an Ag/AgCl (saturated KCl) reference electrode [33,36], for dif-
different applied oxidation potentials, $E_{ap} = +0.5$ V, +0.9 V, and +1.1 V, was also used. All microvolumes were measured using EP-10 and EP-100 Plus Motorized Microliter Pipettes, Rainin Instrument Co. Inc., Woburn, USA. The pH measurements were carried out with a Crison microPH 2001 pH-meter with an Ingold-combined glass electrode. All experiments were done at room temperature ($25 \pm 1^\circ$C). The ultrasound bath used was from Bandelin Sonorex, Germany. The microwave was a Miele, M625EG, Germany.

2.2.2 Voltammetric Parameters and Electrochemical Cells

Voltammetric experiments were carried out using an Autolab potentiostat running with GPES 4.9 software, Metrohm/Autolab, Utrecht, the Netherlands. Measurements were carried out using a GCE, and an Ag/AgCl (3 M KCl) reference electrode, in a 1 mL one compartment electrochemical cell (eDAQ Products, Poland). The GCE was polished using diamond spray (particle size 1 μM, Kement, Kent, UK) before every electrochemical assay. After polishing, the electrode was rinsed thoroughly with Milli-Q water. Following this mechanical treatment, the electrode was subjected to ultrasound treatment at 25°C and constant frequency of 35 kHz during 15 min. After that, the sample was taken out of the ultrasound bath and left at room temperature for 1 h, centrifuged at 3500 rpm during 20 min, and the extract collected in the laboratory flask and stored in the fridge.

The remaining solid sample was again mixed with 3 mL of the extraction solvent and subjected to the same ultrasound treatment for 30 min, left at room temperature for 1 h, centrifuged, and the second extract was collected.

The remaining solid sample was once again extracted with 3 mL of extraction solvent, during 15 min in the ultrasound bath, left to stand for 1 h at room temperature, centrifuged and the third extract was collected.

Finally, the three portions of the $Cp_L$ extracts were mixed and the total volume was measured. Before the HPLC analysis, the extracts were filtered using the 0.45 μm syringe filter (Schleicher & Schuell, Dassel, Germany), and adequately diluted in the mobile phase.

2.4 HPLC Experimental Conditions

The experimental conditions for the HPLC analysis followed programmed sequences of isocratic or gradient elution.

The mobile phase for isocratic elution was 100% (water-methanol-formic acid 80 : 20 + 2 mL formic acid (500 mL)) for 60 minutes.

The mobile phases for gradient elution were: solvent A (water-methanol-formic acid 83 : 16 : 1 by volume) and solvent B (water-methanol-formic acid 68.5 : 30 : 1.5). The programmed sequence was for the first 11 minutes isocratic elution 100% solution A, followed by linear gradient elution for 11–21 minutes from 100% solvent A to 100% solvent B, and followed by isocratic elution for 21–60 minutes solvent B.

Both mobile phases had pH ~2.20. The retention times ($t_r$) obtained depended of the elution mode isocratic or gradient.

The HPLC conditions used were: range = 5 nA, fit = 0.1 s, offs = 10%. The mobile phase flow rate was 1.0 mL min$^{-1}$, and the injected volume was 25 μL. The guard column, the analytical column and the electrochemical flow cell detector were kept in a Faraday cage with the thermostatic oven at 40°C.

The photodiode array detection (PDA) experimental conditions were 190 nm < λ < 600 nm.

2.5 Antioxidant Capacity Evaluation Using the DPPH Method

The radical scavenging activity of $Cp_L$ was measured based on the conversion (decolorization) of stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical by the $Cp_L$’s antioxidants. The ability of the sample to scavenge DPPH radicals was determined by the Brand-Williams assay [12,23]. Briefly, to 2.5 mL of DPPH methanolic solution (90 μM) an aliquot of 0.5 mL of methanol blank control was added in order to reach a final volume of
3.0 mL. The reaction solution was incubated for 30 min in the dark at room temperature and measured at $\lambda = 516$ nm. The total antioxidant activity was expressed as “efficient concentration” or $EC_{50}$, representing the amount of extract to produce 50% of decolorization of DPPH relative to the methanol blank control [12–15,23].

3 Results and Discussion

The ultrasound-assisted treatment, with small ultrasound exposure time combined with the traditional solid-liquid extraction at the room temperature, and the microwave-assisted extraction technique developed to optimize the extraction of the $CpL$ phenolic compounds, were used to reduce the extraction time and degradation processes of flavonoids.

3.1 Detection of Flavonoids in the $CpL$ Extract

The method used in the separation and identification of the flavonoids of $CpL$ flower buds extract was already optimized [30].

![Figure 2](image1.png)

Fig. 2. RP-HPLC of $CpL$ extract, diluted 100 times with isocratic elution and detectors: (A) PDA, and (B) EC with GCE, at $E_{ap} = +0.50$ V vs. ISAAC. Peak identification: (1) delphinidin, (2) myricetin, (3) catechin, (4) kuromanin, (5) epicatechin, (6) callistephin, (7) procyanidin A2, (8) fisetin and (9) rutin.

![Figure 3](image2.png)

Fig. 3. RP-HPLC of $CpL$ extract, diluted 100 times with gradient elution and detectors: (A) PDA, and (B) EC with GCE, at $E_{ap} = +0.50$ V vs. ISAAC. Peak identification: (1) delphinidin, (2) myricetin, (3) catechin, (4) kuromanin, (5) epicatechin, (6) callistephin, (7) procyanidin A2, (8) fisetin and (9) rutin.

The detection by RP-HPLC separation coupled with electrochemical (EC) and photodiode array detection (PDA) detectors was carried out in a comparative study using flavonoid standards before applying the RP-HPLC-EC method to the determination of the flavonoids in the $CpL$ extract, Figure 2 and 3.

The surface area of the electrochemical detectors was very different, consequently using the GCE the extract solution was diluted 100 times, and using the BDDE the extract solution was diluted 133 times.

RP-HPLC-PDA was used to identify and to confirm the elution time of all flavonoid standards, based on their UV/Vis spectra recorded at $\lambda = 280, 350$ and 491 nm, Figures 2A and 3A. Flavan-3-ols, namely catechin, are usually detected at $\lambda = 280$ nm, but catechin, due to its low concentration in the $CpL$ sample extract, could not be detected with the PDA detector.

RP-HPLC-EC analysis using the two procedures, isocratic or gradient elution, enabled the identification of nine flavonoids in the $CpL$ extract: catechin, delphinidin, fisetin, myricetin, epicatechin, kuromanin, rutin, callistephin and procyanidin A2, Figures 2B and 3B. The electro...
trochemical detection allowed the identification of the flavonoid standards at much lower concentrations than the PDA and without interferences. These results showed the excellent electrochemical detection sensitivity, and suitability for the detection of low levels of electroactive phenolic compounds.

A RP column was used for the chromatographic separation, and the retention times \( t_r \) of the flavonoid standards was determined. The most polar compounds, delphinidin, myricetin catechin, kuromanin and epicatechin, were eluted early, followed by flavonols, fisetin and rutin. The retention times \( t_r \) obtained for all flavonoid standard compounds analyzed are given in Table 1.

The separation of the phenolic compounds in isocratic conditions was used because baseline drift occurred when gradient elution was employed with EC and a long time for baseline stabilization was necessary.

Gradient elution was only employed to change the polarity of the mobile phase, i.e. to change from solvent A to solvent B, with the aim to accomplishing the complete elution of the mixture of the CpL extract.

Catechin is widely used as a reference standard in antioxidant comparative studies [21,26], in part due to its stability, so in this work catechin was also selected as a reference standard. Using the same experimental conditions a catechin standard chromatogram, Figure 4I, was compared with the CpL extract chromatogram, Figure 4II, and the CpL extract spiked with 25 \( \mu \)L of \( 10^{-6} \) M catechin standard chromatogram, Figure 4III.

As expected, an increase of the peak intensity at \( t_r = 7.2 \) min (gradient), at the retention time of catechin was observed, Figure 4III, so the catechin presence in the CpL extract composition was confirmed.

The HPLC-EC analysis of CpL extract was carried out using the two different electrochemical detectors: the wall-jet cell with the CGE and the thin-layer cell with the BDDE, at different applied potentials, from \( E_{ap} = +0.5 \) V till \( E_{ap} = +1.1 \) V, Figure 5. The isocratic elution mode was used because the chromatograms always presented better defined peaks.

The HPLC with the BDDE gave a better separation and definition for the CpL extract peaks compared with the HPLC with GCE. The HPLC with the BDDE enabled the application of a higher oxidation potential and

### Table 1. RP-HPLC-EC retention times \( t_r \) for flavonoid standards.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Isocratic ( t_r ) (min)</th>
<th>Gradient ( t_r ) (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Delphinidin (1)</td>
<td>3.5</td>
<td>–</td>
</tr>
<tr>
<td>Myricetin (2)</td>
<td>6.4</td>
<td>7</td>
</tr>
<tr>
<td>Catechin (3)</td>
<td>6.5</td>
<td>7.2</td>
</tr>
<tr>
<td>Kuromanin (4)</td>
<td>9.9</td>
<td>13</td>
</tr>
<tr>
<td>Epicatechin (5)</td>
<td>12.1</td>
<td>13.9</td>
</tr>
<tr>
<td>Callistephin (6)</td>
<td>–</td>
<td>18.3</td>
</tr>
<tr>
<td>Procyanidin A2 (7)</td>
<td>28.8</td>
<td>26</td>
</tr>
<tr>
<td>Fisetin (8)</td>
<td>–</td>
<td>37</td>
</tr>
<tr>
<td>Rutin (9)</td>
<td>–</td>
<td>43</td>
</tr>
</tbody>
</table>

![Fig. 4. RP-HPLC-EC with GCE of the CpL extract, diluted 100 times, and catechin standard, with gradient elution, at \( E_{ap} = +0.5 \) V vs. ISAAC: (I) \( 10^{-6} \) M catechin standard, (II) CpL extract and (III) CpL extract with 25 \( \mu \)L of \( 10^{-6} \) M catechin standard. Peak \( t_r \) identification: (3) catechin.](image)

![Fig. 5. RP-HPLC-EC of CpL extract, isocratic elution: with GCE vs. ISAAC diluted 100 times, and with BDDE vs Ag/AgCl diluted 133 times, at different oxidation potentials. Peak \( t_r \) identification: (1) delphinidin, (2) myricetin, (3) catechin, (4) kuromanin, (5) epicatechin and (7) procyanidin A2.](image)
therefore some compounds in the CpL extract that are oxidized at a higher oxidation potential were detected.

3.2 CpL Extract Total Antioxidant Capacity Voltammetric and Spectrometric Evaluation

The voltammetric and spectrometric evaluation of CpL extract total antioxidant capacity was investigated. Antioxidant compounds exhibit electrochemical behavior; therefore electroanalysis is an important tool to evaluate their antioxidant capacity in foodstuffs. Voltammetric techniques such as cyclic voltammetry and differential pulse voltammetry have become alternatives to traditional spectrophotometric methods due to the relevance of the information provided, the simplicity of the assay, and the ability to process colored and turbid samples.

The electrochemical oxidation of a catechin standard and a CpL extract sample were studied in pH 1.9 and pH 6.9 by DP voltammetry. The electrochemical oxidation mechanism of catechin was studied [37], Figure 6. Both catechin and CpL extract presented a similar oxidation behavior, in two oxidation steps, peaks 1a and 2a, Figure 7.

DP voltammograms in 10 μM catechin, in 0.1 M phosphate buffer pH 6.9, presented two oxidation peaks, at a lower potential peak 1a, $E_{p1a} = +0.19$ V, and at a higher potential peak 2a, $E_{p2a} = +0.52$ V, Figure 7B. The peak 1a corresponds to the reversible oxidation of the $\cdot$OH groups in the B-ring and peak 2a corresponding to the irreversible oxidation of $\cdot$OH group in the C-ring of catechin [37], Figure 6.

In acid media, pH 1.9, Figure 7A, the catechin peaks 1a and 2a oxidation potentials were shifted to more positive potentials, $E_{p1a} = +0.51$ V and $E_{p2a} = +0.89$ V, and peak 1a current decreased, but peak 2a current did not change.

The polyphenols on the CpL extract were oxidized and the response was the sum of the oxidation of the different polyphenolic species present in CpL extract.

DP voltammograms for different concentrations of CpL extracts (5/1000 μL, 10/1000 μL, and 35/1000 μL) showed very strong adsorption on the glassy carbon electrode. The concentration chosen was 5/1000 μL, in 0.1 M phosphate buffer pH 6.9, and two oxidation peaks, at low potential peak 1a, $E_{p1a} = +0.19$ V, and at high potential peak 2a, $E_{p2a} = +0.66$ V, occurred, Figure 7B. In acid media, pH 1.9, the peak oxidation potentials were peak 1a, $E_{p1a} = +0.51$ V, and peak 2a, $E_{p2a} = +0.88$ V, but the all peak currents were higher at pH 1.9.

Fig. 6. Oxidation mechanism of catechin peak 1a [37].

![Fig. 6. Oxidation mechanism of catechin peak 1a [37].](image)

![Fig. 7. DP voltammograms of (—) 10 μM catechin and (···) CpL extract, at GCE: (A) pH 1.9 and (B) pH 6.9. Scan rate 5 mV/s.](image)
The oxidation behavior of the CpL extract was similar to the behavior of catechin for both pHs, Figure 7.

The peak 1a for CpL extract with the lowest oxidation potential was considered as the “high antioxidant power” peak, and the peak 2a, with the highest oxidation potential was considered the “low antioxidant power” peak. The first peak of CpL extract also occurred for catechin (oxidation of related flavan-3-ols) and the second peak occurred for catechin and other phenolic compounds present in the CpL extract. The high antioxidant power assigned to the CpL extract is mainly attributed to a predominant flavonoid class, flavan-3-ols.

The electrochemical index (EI) for catechin and for the CpL extract, for pH 1.9 and pH 6.9, based on the peak potential and peak current of the highest peak, peak 1a, was calculated, Table 2. Catechin was chosen as a flavonoid standard because it is a natural electroactive compound with important antioxidant activity and with a high EI value.

Catechin presented, for both experimental conditions, EI = 0.943 μAV−1 for pH 1.9 and EI = 4.001 μAV−1 for pH 6.9, a higher value for EI than the CpL extract, EI = 0.388 μAV−1 for pH 1.9 and EI = 1.082 μAV−1 for pH 6.9, Table 2.

The “efficient coefficient” or EC50 value, obtained by the DPPH’ method, is defined as the concentration of substrate that causes 50% loss of DPPH’ activity [13].

The EC50 represents the total phenols concentration (total antioxidant concentration) of the CpL extract necessary to reduce 50% of DPPH’ concentration, so it is inversely proportional to EI, Table 2.

For the CpL extract EC50 = 0.122 mgmL−1, much higher than for catechin, EC50 = 1.743 × 10−5 mgmL−3, in agreement with catechin higher antioxidant capacity.

The EC50 values presented in Table 2, for both catechin and the CpL extract, are in agreement with their EI values. The CpL extract contained in its composition other chemicals, besides flavonoids, including alkaloids, proteins, tannins, steroids, phenols, carbohydrates and terpenoids [8,10], that affect the evaluation of the total phenolic concentration.

As expected the EI value of the CpL extract, which represented the total phenolics concentration obtained with electrochemical techniques, was lower than the EI of catechin, due to the complex CpL extract composition.

Table 2. EC50 and EI for CpL flower buds extract and catechin standards.

<table>
<thead>
<tr>
<th></th>
<th>EC50 (mgmL−1)</th>
<th>EI (μAV) pH 6.9</th>
<th>EI (μAV) pH 1.9</th>
</tr>
</thead>
<tbody>
<tr>
<td>CpL extract</td>
<td>0.122</td>
<td>1.082</td>
<td>0.388</td>
</tr>
<tr>
<td>Catechin</td>
<td>1.743 × 10−5</td>
<td>4.001</td>
<td>0.943</td>
</tr>
</tbody>
</table>

4 Conclusions

The separation, detection, and identification in a single run, by direct injection of the Calligonum polygonoides Linnaeus dried immature flower buds extract, using a RP-HPLC-EC, of nine different flavonoids: catechin, delphinidin, fisetin, myricetin, epicatechin, kuromanin, rutin, callistephin and procyanidin A2, was performed. The total antioxidant capacity of the CpL was quantified, using DP voltammetry and the DPPH’ spectrophotometric methodology. The electrochemical detection has excellent sensitivity and selectivity, and should be widely used to analyze important electroactive species such as antioxidants.

The flower buds of Calligonum polygonoides are loaded with pigmented substances and are aromatic. These studies reveal that the Phog or Phogla may be a good source of antioxidants and anti-stress compounds. The Phog plant has a number of properties that help users fight the types of stresses induced by intense heat, famine and water scarcity.

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