Antitryptical, anticoagulant and hemagglutinating activities of *Eucalyptus* sp. seeds [version 1; referees: awaiting peer review]

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Abstract

Background: Plant biodiversity has great value for science being an inexhaustible source for new bioactive molecules capable of offering environmentally friendly and innovative solutions for various areas of the industry. The scientific community has increased their interest in the study of plant species in the search of new molecules and to determine their mechanisms of action. Plant seeds are natural sources of bioactive compounds, such as carbohydrates, lipids and proteins with special focus on enzymatic inhibitors which protect them against digestive enzymes of phytopathogens and lectins that play an important role on carbohydrate signalization and metabolism during germination. The objective of the present study was to evaluate and describe the protein profile and to test the hemagglutinating, hemolytic and anticoagulant activities, as well as the antitryptic effect of extracts and fractions obtained from seeds of *Eucalyptus* species.

Methods: The crude protein extract was obtained from the seed of *Eucalyptus* sp. with 0.02 M sodium phosphate buffer, at pH 6.6, and fractionated using ammonium sulfate in order to study its antitryptical properties as well as the capacity of hemagglutination and influence on hemostasis.

Results: The crude extract showed a high effectiveness for trypsin inhibition. For hemagglutinating activity, the ammonium sulfate fraction 0-30% presented better activity, while no hemolytic activity was present in the obtained fractions. For anticoagulation assay, the fraction 0-30% showed better results.

Conclusions: Taken together, the obtained results demonstrate the biotechnological potential of *Eucalyptus* sp. seeds, although further study is still necessary to better isolate as well as describe the bioactive compounds.

Keywords

Eucalyptus sp., seed, trypsin inhibitor.
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Introduction
The high biodiversity of plants has led to them becoming of increasing interest to research communities due to their potential for providing new bioactive molecules with new mechanisms of action (Viegas et al., 2006). Among the most important studied plant structures, seed extracts have demonstrated potential biological activities such as protease inhibition, hemagglutinating, antibacterial and anticoagulant activities (Otielo & Analo, 2012).

The seed is the structure of a plant responsible for the propagation, and dispersion of plants in the environment, as well as nourishing and protecting the embryo at the first critical stages of germination and establishment in soil (Mello et al., 2010). To fulfill such functions these organs require a true arsenal of molecules, such as carbohydrates, lipids, amino acids and proteins (Banik et al., 2018; Mello et al., 2010).

Eucalyptus belongs to Myrtaceae family (Otielo & Analo, 2012) and some species of this genus are used in the treatment of certain bacterial or fungal infections in humans. Eucalyptus monoculture provides distinct products, such as wood, charcoal, resins, plywood, cellulose ethanol, cellulose and paper (Takahashi et al., 2004). The present study had as objective to evaluate the protein profile and to test the hemagglutinating, hemolytic and anticoagulant activities, as well as the antipr inflammatory effect of the crude extracts and fractions obtained from the seeds of Eucalyptus sp.

Methods
Protein extraction and fractionation of Eucalyptus sp. seeds
The seeds used in the present study were donated by the seed bank of the National Forest (Flona) of Nísia Floresta, located in the district of Nísia Floresta, Rio Grande do Norte, Brazil. They were powdered using refrigerated mill (TE® 631/2) until obtaining a fine flour. The ground seeds were then homogenized in 0.02 M sodium phosphate buffer, pH 6.0, under constant stirring using magnetic stirrer (Solab SL-91/A) for 4 hours at 4°C. The homogenate was centrifuged (Hettich® MIKRO 200/200R) at 10,000 x g for 30 minutes at 4°C. The supernatant was termed crude extract (EB). EB was sequentially fractionated in two steps (0–30% named after F1 and 30–60% named as F2) with ammonium sulfate at 30% (w/v) then 60% (w/v) and further centrifuged at 10,000 x g for 30 minutes at 4°C. The pellet was resuspended in distilled water and dialyzed against the same solvent. Protein quantification was performed according to the method described by Bradford (Bradford, 1976) with adaptations for microplate assay. Plate reading was performed at 595 nm using EPOCH™ (Biotek®) microplate reader.

SDS-PAGE and protein weight estimation
The electrophoretic protein pattern of Eucalyptus sp. fractions were observed by SDS-PAGE 12.5% (SDS-PAGE kit 1615100, Bio-Rad®) according to Laemmli (Laemmli, 1970). The protein bands also were visualized by silver staining and the approximate molecular mass were estimated by SDS-PAGE using as reference the molecular weight (Kaleidoscope™, Bio-Rad®) and migration pattern of Bovine Serum Albumin (BSA) (code A9418, Sigma-Aldrich®).

Hemagglutinating activity
Human red blood cells (from blood bags generously donated by the Hemocentro Dalton Cunha, Rio Grande do Norte, Brazil) from different types (A, B and O) treated with papain or trypsin (both of them at 0.5 mg/mL) were incubated with serial dilutions of EB, F1 or F2 in saline solution (NaCl 0.15 M) in a 96-well plate, at a ratio of 1:1. The plate was incubated for 1 hour (at pH 7.4 and 22°C), and a negative control (saline solution and red blood cells) was performed for further comparison. The degree of agglutination was visually analyzed and the titre expressed in hemagglutination unit (U.H.), which is defined as the inverse of the highest dilution where Red Blood Cells (RBCs) agglutination was observed.

Hemolytic activity
RBCs were separated from the plasma by sedimentation and washed three times with saline solution. Then, 100 μL of the red blood cell suspension were incubated with 100 μL of the samples (EB, F1 or F2) for 60 minutes at 25°C. For positive control, 100 μL of RBCs suspension was incubated with 100 μL of 1% Triton X-100, while 100 μL of saline was incubated with same volume of RBCs suspension for negative control. After incubation, the reaction mixture was centrifuged (Hettich® MIKRO 200/200R) at 3,200 x g for 5 minutes at 25°C. Aliquots of 100 μL of supernatants were transferred to 96-well plates and analyzed by spectrophotometry with readings at 405 nm (Pharmacia Biotec® Ultrospec 2100 pro). The mean and standard deviation was determined by three replicate assays.

Trypsin inhibition assay
In order to evaluate the capacity of trypsin inhibition by Eucalyptus sp. seeds protein, the test was performed according to Xavier-Filho (Xavier-Filho et al., 1989), where aliquots of 10 μL of bovine trypsin (code T8802, Sigma-Aldrich®) solution (0.3 mg/ml in 50 mM tris-HCl buffer, pH 7.5) were preincubated with 120 μL of 2.5 mM HCl as well as 320 μL of 50 mM Tris-HCl buffer, pH 7.5 and 50 μL of samples from Eucalyptus sp. for 15 minutes at 37°C. After this period, the reaction was started by adding 200 μL of 1% (w/v) Azocasein (code A 2765, Sigma-Aldrich®) solution for another 30 min. The reaction was finally stopped by adding 300 μL of 20% NaOH. The effect of the fractions on the proteolytic activity was monitored by spectrophotometer (Pharmacia Biotec® Ultrospec 2100 pro) in the 410 nm wavelength.

Anticoagulant activity evaluation
Human blood was added to tubes containing sodium citrate and centrifuged at 3,200 x g for 5 minutes at room temperature for separation of plasma and red blood cells. This assay was performed with serial dilutions of the samples (EB, F1 and F2) in 0.15 M PBS buffer, pH 7.4. Aliquots of 90 μL plasma were mixed with 10 μL of samples in different protein amounts (100, 25, 12.5, 6.25, 3.12, 1.56, 0.78, 0.39 μg) and incubated for
3 minutes at 37°C. Then, 100 μL of 25 mM calcium chloride was added, after 1 hour the presence or not of coagulation was observed. For the negative control, 0.15 M PBS buffer, pH 7.4 with plasma was used. The tests were adapted from the United States Pharmacopeia (1965).

Results and discussion

SDS-PAGE and protein weight estimation

The electrophoretic profile showed two major bands, one near the region corresponding to the molecular weight of bovine serum albumin (BSA), in this case, used as a marker with a known molecular mass of 66 kDa, and a second band presenting a lower molecular weight, analyzed by linear regression for the used molecular marker as performed by Dos Santos (Dos Santos et al., 2018) (Figure 1).

Evaluation of trypsin inhibition

EB and fractions were tested for their capacity to inhibit the activity of trypsin, a serine protease. The inhibition of trypsin was calculated based on the mass of protein estimated by the Bradford method in each sample. The results showed higher specific inhibitory activity for F1 when compared to the other fractions (Table 1). Since just a small group of proteins with same shared hydrophobicity indices are equally precipitated on each ammonium sulfate fractionation step, it increases the specific activity of proteins (de Oliveira et al., 2018) leading to a higher activity as previously described (Kunitz & Northrop, 1936; Oliveira et al., 2009).

Anticoagulant activity

The EB and fractions (F1 and F2) obtained from the seeds were tested for anticoagulant activity and the results obtained are presented in Table 2. Anticoagulant activity was identified in all tested samples, with the highest activity exhibited by F1 with 12.5 μg of protein. Protease inhibitors, such as those studied in the present work, have the potential to inhibit blood coagulation cascade proteases arising as potential hemostasis modulators with clinical application on the treatment of blood clots (Harish & Uppuluri, 2018; Tagnon & Soulier, 1946).

Hemagglutinating activity

The extract and fractions were tested with erythrocytes A, B and O, treated separately, with papain and trypsin, in triplicate (Figure 2 and Figure 3). EB showed maximum titer 512 U.H. of hemagglutination for all blood types treated with papain (code P4762, Sigma-Aldrich®), and a minimum of 64 U.H. for blood B untreated with enzymes. The F1 fraction had a maximum titer of 1024 U.H for A and O erythrocytes treated with papain and trypsin, presenting the same titer for papain-treated B erythrocytes. The lowest titer of hemagglutination obtained for F1 was 256 U.H for untreated B and O types erythrocytes as described for other studies on protein seeds fractionation (Braga et al., 2015; Vodkin & Raikhel, 1986).

Hemolytic activity

None of the tested samples showed hemolytic activity for any blood type even at concentrations as high as 300 μg/mL (Table 3).

![Figure 1. SDS-PAGE profile of Eucalyptus sp. seeds proteins revealed with silver nitrate. M - Marker; EB: Crude extract, F1: Fraction precipitated with ammonium sulphate 0–30%; F2: Precipitated fraction with ammonium sulphate 30–60%.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Total inhibitory activity (IU)</th>
<th>Total protein (mg)</th>
<th>Specific inhibitory activity (IU/mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EB</td>
<td>27.0</td>
<td>0.02</td>
<td>1,350</td>
</tr>
<tr>
<td>F1</td>
<td>19.8</td>
<td>0.01</td>
<td>1,980</td>
</tr>
<tr>
<td>F2</td>
<td>14.9</td>
<td>0.01</td>
<td>1,490</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Sample protein content (μg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample</td>
</tr>
<tr>
<td>EB</td>
</tr>
<tr>
<td>F1</td>
</tr>
<tr>
<td>F2</td>
</tr>
</tbody>
</table>
Table 3. Evaluation of the hemolytic effect of the crude extract (EB), 0–30% fractions (F1) and 30–60% fraction (F2) on human red blood cells. Saline phosphate buffer (PBS) and 1% Triton X-100, were respectively used as negative (-) and positive (+) control for hemolytic activity.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Type A Blood</th>
<th>Type B Blood</th>
<th>Type O Blood</th>
<th>PBS</th>
<th>Triton X-100</th>
</tr>
</thead>
<tbody>
<tr>
<td>EB</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>F1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>F2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

Conclusions
In summary, this study described the extraction and fractionation of proteins from Eucalyptus sp. seeds with antitryptic and hemagglutinating activities, suggesting the possible occurrence of bioactive proteins like lectin and protease inhibitors. In addition, none sample showed hemolytic activity against human erythrocytes. Taken together, the obtained results demonstrate...
the biotechnological potential of *Eucalyptus* sp. seeds, being still necessary to perform further studies in order to better isolate as well as describe the bioactive compounds to be detected.

**Data availability**

**Underlying data**

Open Science Framework: The underlying data generated in the present study, https://doi.org/10.17605/OSF.IO/KFAEX (Santos, 2018).

Data are available under the terms of the Creative Commons Zero “No rights reserved” data waiver (CC0 1.0 Public domain dedication).

- SDS-PAGE profile of *Eucalyptus* sp. seeds proteins revealed with silver nitrate

- Raw data for Figure 2 and Figure 3
- Protein quantification
- Hemolytic Assay
- Trypsin activity

**Grant information**

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**References**

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