RESEARCH ARTICLE

Antitryptical, anticoagulant and hemagglutinating activities of *Eucalyptus* sp. seeds [version 1; peer review: 2 approved with reservations]

Yago Queiroz dos Santos, Gabriella Silva Campos Carelli, Bruno Oliveira de Veras, Virgínia Cunha Batista, Anderson Felipe Jácome de França, Márcia Vanusa da Silva, Elizeu Antunes dos Santos

1Institute of Tropical Medicine, Federal University of Rio Grande do Norte, Natal, Rio Grande do Norte, Brazil
2Department of Biochemistry, Federal University of Rio Grande do Norte, Natal, Rio Grande do Norte, Brazil
3Department of Biochemistry, Federal University of Pernambuco, Recife, Pernambuco, Brazil

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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, hemolytic and anticoagulant activities, as well as the antitryptic effect of extracts and fractions obtained from seeds of *Eucalyptus* species.

**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.
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 (Otieno & 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 (Otieno & 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, cellulosic 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 antitryptic 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 its 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% TCA. The reaction mixture was centrifuged for 12 min at 10,000 x g and 500 μL aliquot of the supernatant were alkalized with 500 μL 2N 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.](image)

**Table 1.** Trypsin inhibitory activity of the crude extract (EB) and 0–30% fractions (F1) and 30–60% fraction (F2) of Eucalyptus sp. seeds.

| Sample | Total inhibitory activity (IU) | Total protein (mg) | Specific inhibitory activity (IU/mg of protein) |
|--------|-------------------------------|--------------------|-----------------------------------------------|
| EB     | 27.0                          | 0.02               | 1,350                                         |
| F1     | 19.8                          | 0.01               | 1,980                                         |
| F2     | 14.9                          | 0.01               | 1,490                                         |

**Table 2.** Anticoagulant activity of Eucalyptus sp. seeds crude extract (EB), 0–30% fractions (F1) and 30–60% fraction (F2), which were tested in serial dilution. The amount of protein present per dilution is expressed in μg. (-) Absence of coagulation; (+) Presence of coagulation.

| Sample | Sample protein content (μg) |
|--------|-----------------------------|
| EB     | 100 | 50 | 25 | 12.5 | 6.25 | 3.12 | 1.56 | 0.78 | 0.39 |
| F1     | -   | -  | -  | +   | +   | +   | +   | +   | +   |
| F2     | -   | -  | -  | +   | +   | +   | +   | +   | +   |
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.

| Sample | Type A Blood | Type B Blood | Type O Blood | PBS | Triton X-100 |
|--------|--------------|--------------|--------------|-----|--------------|
| EB     | -            | -            | -            | -   | +            |
| F1     | -            | -            | -            | -   | +            |
| F2     | -            | -            | -            | -   | +            |

Figure 2. Haemagglutinating activity of *Eucalyptus* sp. seeds proteins crude extract (EB) obtained with 0.02 M sodium phosphate buffer, pH 6.6. titer expressed in hemagglutination units (UH).

Figure 3. Hemagglutinating activity of *Eucalyptus* sp. seeds proteins fraction 0–30% (F1) obtained from extraction with 0.02 M sodium phosphate buffer, pH 6.6. titer expressed in hemagglutination units (UH).

Conclusion
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](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

### Grant information

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The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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Version 1

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Maria G Pereira
Laboratory of Bioactive Polysaccharides, Universidade Estadual do Ceará, Fortaleza, Ceará, Brazil

The article, submitted by Queiroz dos Santos et al., aims to extract (0.02 M sodium phosphate buffer, pH 6.0) and fractionate the proteins [with ammonium sulfate at 30% (w/v) – F1 and at 30–60% (w/v) - F2] of seeds from Eucalyptus sp, and to evaluate the protein pattern of the fractions by SDS-PAGE 12.5% electrophoresis, as well its hemagglutinant, hemolytic, trypsin inhibition, anticoagulant activities. This one needs to provide a major review and English review.

- Is the work clearly and accurately presented and does it cite the current literature?
  Response: in part. See comments below.

- Is the study design appropriate and is the work technically sound?
  Response: in part. See comments below.

- Are sufficient details of methods and analysis provided to allow replication by others?
  Response: should be better detailed to replication.

- If applicable, is the statistical analysis and its interpretation appropriate?
  Response: it is important to include.

- Are all the source data underlying the results available to ensure full reproducibility?
  Response: in part. See comments below.

- Are the conclusions drawn adequately supported by the results?
  Response: yes, are in accordance with demonstrated results.

Major Reviews

Introduction:

1. Question: Are there studies involving some biological activities, more related to the present study, described for Eucalyptus genus? If positive, include in the introduction and discussion.

2. Why evaluate these biological activities for Eucalyptus? Improve this point.

Methods: should be better detailed to allow replication.

1. Question: Is there an exsiccate number of this plant? Botanical Identification?
2. How much seeds and of powder were used?

3. Sentence: ...“The ground seeds were then homogenized in 0.02 M sodium phosphate buffer, pH 6.0” - what proportion?

4. Sentence: “crude extract (EB)” - correct acronym.

5. Sentence: “method described by Bradford (Bradford, 1976) with adaptations for microplate assay” – cite reference of the microplate method.

6. Hemagglutination unit (U.H.) - correct acronym.

7. Hemagglutinating activity: cite reference of the method.

8. Hemolytic activity: cite reference of the method;

9. Include statistical analysis;

Results and Discussion

Table 1:
- EB, F1 and F2: to present the yields;
- Are there carbohydrates in these samples? It is important to dose.
- Discuss why the difference in specific activity between F1 and F2 (i.e. yield, total protein, degree of purity);
- Is important to add a negative control;
- Include in discussion the relation between the results obtained in SDS-PAGE and trypsin inhibitory activity;

Table 2:
- Improve the presentation of Table 2, the current format is confusing;
- Include the blood clotting time obtained in presence of EB, F1 and F2;
- Include negative and positive controls;
- Thrombin time (common pathway) and prothrombin (extrinsic pathway) has not been evaluated? Discuss it.

Figure 1:
- Include MW of the others markers protein;
- Which component present in the samples is under BSA? Identify its MW in figure;
- EB and F1 electrophoretic profile is different from F2 fraction? Explain.
- Include in discussion what's relation between F1 electrophoretic profile and its higher
specific inhibitory activity?

- Electrophoresis: these MW obtained is characteristic of other protease inhibitors?

Figures 2 and 3:
- What's the importance of enzymatic treatment with papain and trypsin? Why the results were higher with treated blood? Include in discussion;

- F2 was not tested in haemagglutinating activity? Include in the discussion the justification for this;

- Why did the blood type "O" treated with trypsin not increase HU? Only treated with papain, is there any reason for that?

References:
- Standardize according to the rules of F1000Research.

Is the work clearly and accurately presented and does it cite the current literature?
Partly

Is the study design appropriate and is the work technically sound?
Partly

Are sufficient details of methods and analysis provided to allow replication by others?
Partly

If applicable, is the statistical analysis and its interpretation appropriate?
No

Are all the source data underlying the results available to ensure full reproducibility?
Partly

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Biochemistry.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.
Devaraja Sannaningaih
Department of Studies and Research in Biochemistry and Centre for Bioscience and Innovation, Tumkur University, Tumkur, India

Queiroz dos Santos et al. made an attempt here. I really appreciate their effort but there are some points to mention.

1. Objective of the study is not clear.

2. Results are not presented well.

3. Discussion part is also not up to the mark.

4. Lot of grammatical errors.

5. SDS-PAGE is good, rest of the graphs are of poor quality.

Thus in its present form it is difficult to accept, but it can be reconsidered as a short communication rather than a full length paper with major revision.

- Present the results and discussion separately.

- Give the microscopic image of hemagglutination and hemolysis.

- Better try anticoagulant activity in-vivo.

- Carry out APTT and PT assay

**Is the work clearly and accurately presented and does it cite the current literature?**
Yes

**Is the study design appropriate and is the work technically sound?**
Yes

**Are sufficient details of methods and analysis provided to allow replication by others?**
Yes

**If applicable, is the statistical analysis and its interpretation appropriate?**
Yes

**Are all the source data underlying the results available to ensure full reproducibility?**
No

**Are the conclusions drawn adequately supported by the results?**
Partly

**Competing Interests:** No competing interests were disclosed.

**Reviewer Expertise:** Protein chemistry, platelet biology, thrombosis and hemostasis, nanotechnology.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

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