RESEARCH ARTICLE

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

Yago Queiroz dos Santos ^{1,2}, Gabriella Silva Campos Carelli^{1,2},
Bruno Oliveira de Veras³, Virgínia Cunha Batista²,
Anderson Felipe Jácome de França ^{1,2}, Márcia Vanusa da Silva³,
Elizeu Antunes dos Santos^{1,2}

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

V1 First published: 08 Jan 2019, 8:28
<https://doi.org/10.12688/f1000research.17080.1>
Latest published: 08 Jan 2019, 8:28
<https://doi.org/10.12688/f1000research.17080.1>

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

Open Peer Review

Approval Status ? ?

	1	2
version 1	?	?
08 Jan 2019	view	view

1. **Devaraja Sannanigaiah**, Tumkur University, Tumkur, India

2. **Maria G Pereira** , Universidade Estadual do Ceará, Fortaleza, Brazil

Any reports and responses or comments on the article can be found at the end of the article.

study is still necessary to better isolate as well as describe the bioactive compounds.

Keywords

Eucalyptus sp., seed, tripsin inhibitor.

Corresponding author: Yago Queiroz dos Santos (yagoqs@hotmail.com)

Author roles: **Queiroz dos Santos Y:** Methodology, Writing – Original Draft Preparation, Writing – Review & Editing; **Silva Campos Carelli G:** Conceptualization, Investigation, Methodology, Writing – Original Draft Preparation, Writing – Review & Editing; **Oliveira de Veras B:** Software, Writing – Original Draft Preparation, Writing – Review & Editing; **Cunha Batista V:** Data Curation, Investigation; **Felipe Jácome de França A:** Conceptualization, Visualization; **Vanusa da Silva M:** Conceptualization, Supervision, Visualization; **Antunes dos Santos E:** Project Administration, Supervision, Writing – Review & Editing

Competing interests: No competing interests were disclosed.

Grant information: This work was supported by the Federal University of Rio Grande do Norte as well as by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

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

Copyright: © 2019 Queiroz dos Santos Y *et al.* This is an open access article distributed under the terms of the [Creative Commons Attribution License](#), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

How to cite this article: Queiroz dos Santos Y, Silva Campos Carelli G, Oliveira de Veras B *et al.* **Antitryptical, anticoagulant and hemagglutinating activities of *Eucalyptus* sp. seeds [version 1; peer review: 2 approved with reservations]** F1000Research 2019, 8:28 <https://doi.org/10.12688/f1000research.17080.1>

First published: 08 Jan 2019, 8:28 <https://doi.org/10.12688/f1000research.17080.1>

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. M - Marker; EB: Crude extract, F1: Fraction precipitated with ammonium sulphate 0–30%; F2: Precipitated fraction with ammonium sulphate 30–60%.

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)							
	100	50	25	12.50	6.25	3.12	1.56	0.78
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).

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	-	-	-	-	+

which makes possible an eventual intravenous administration requiring, nonetheless, a further cytotoxicity studies in order to evaluate a proper pharmacological concentration, since same property was described for others trypsin inhibitor molecules (Sintsova *et al.*, 2018).

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

This work was supported by the Federal University of Rio Grande do Norte as well as by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

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

References

- Banik S, Biswas S, Karmakar S: **Extraction, purification, and activity of protease from the leaves of *Moringa oleifera*** [version 1; referees: 2 approved, 1 approved with reservations]. *F1000Res*. 2018; 7: 1151.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Bradford MM: **A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding.** *Anal Biochem*. 1976; 72(1-2): 248–254.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Braga AA, Rodrigues e Lacerda R, Medeiros GK, *et al.*: **Antibacterial and hemolytic activity of a new lectin purified from the seeds of *Sterculia foetida* L.** *Appl Biochem Biotechnol*. 2015; 175(3): 1689–1699.
[PubMed Abstract](#) | [Publisher Full Text](#)
- de Oliveira CFR, Oliveira CT, Taveira GB, *et al.*: **Characterization of a Kunitz trypsin inhibitor from *Enterolobium timbouva* with activity against *Candida* species.** *Int J Biol Macromol*. 2018; 119: 645–653.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Harish BS, Uppuluri KB: **Potential Anticoagulant Activity of Trypsin Inhibitor Purified from an Isolated Marine Bacterium *Oceanimonas* Sp. BPMS22 and its Kinetics.** *Mar Biotechnol (NY)*. 2018; 20(6): 780–791.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Kunitz M, Northrop JH: **ISOLATION FROM BEEF PANCREAS OF CRYSTALLINE TRYPSINOGEN, TRYPSIN, A TRYPSIN INHIBITOR, AND AN INHIBITOR-TRYPSIN COMPOUND.** *J Gen Physiol*. 1936; 19(6): 991–1007.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Laemmli UK: **Cleavage of structural proteins during the assembly of the head of bacteriophage T4.** *Nature*. 1970; 227(5259): 680–685.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Mello JIDO, Barbedo CJ, Salatino A, *et al.*: **Reserve carbohydrates and lipids from the seeds of four tropical tree species with different sensitivity to desiccation.** *Braz Arch Biol Technol*. 2010; 53(4): 889–899.
[Publisher Full Text](#)
- Oliveira AS, Migliolo L, Aquino RO, *et al.*: **Two Kunitz-type inhibitors with activity against trypsin and papain from *Pithecellobium dumosum* seeds: purification, characterization, and activity towards pest insect digestive enzyme.** *Protein Pept Lett*. 2009; 16(12): 1526–1532.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Otieno NE, Analco C: **Local indigenous knowledge about some medicinal plants in and around Kakamega forest in western Kenya** [version 2; referees: 2 approved]. *F1000Res*. 2012; 1: 40.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Dos Santos YQ, de Veras BO, de França AFJ, *et al.*: **A New Salt-Tolerant Thermostable Cellulase from a Marine *Bacillus* sp. Strain.** *J Microbiol Biotechnol*. 2018; 28(7): 1078–1085.
[PubMed Abstract](#)
- Santos YQd: **Raw Data.** *OSF*. 2018.
<http://www.doi.org/10.17605/OSF.IO/KFAEX>
- Sintsova O, Gladkikh I, Chausova V, *et al.*: **Peptide fingerprinting of the sea anemone *Heteractis magnifica* mucus revealed neurotoxins, Kunitz-type proteinase inhibitors and a new β -defensin α -amylase inhibitor.** *J Proteomics*. 2018; 173: 12–21.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Tagnon HJ, Soulier JP: **Anticoagulant activity of the trypsin inhibitor from soya bean flour.** *Proc Soc Exp Biol Med*. 1946; 61: 440.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Takahashi T, Kokubo R, Sakaino M: **Antimicrobial activities of eucalyptus leaf extracts and flavonoids from *Eucalyptus maculata*.** *Lett Appl Microbiol*. 2004; 39(1): 60–64.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Viegas C Jr, da Silva Bolzani V, Barreiro EJ: **Os produtos naturais e a química medicinal moderna.** *Química Nova*. 2006; 29(2): 326–337.
[Publisher Full Text](#)
- Vodkin LO, Raikhel NV: **Soybean lectin and related proteins in seeds and roots of le and le soybean varieties.** *Plant Physiol*. 1986; 81(2): 558–65.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Xavier-Filho J, Campos FAP, Ary MB, *et al.*: **Poor correlation between the levels of proteinase inhibitors found in seeds of different cultivars of cowpea (*Vigna unguiculata*) and the resistance/susceptibility to predation by *Callosobruchus maculatus*.** *J Agr Food Chem*. 1989; 37(4): 1139–1143.
[Publisher Full Text](#)

Open Peer Review

Current Peer Review Status: ? ?

Version 1

Reviewer Report 29 June 2020

<https://doi.org/10.5256/f1000research.18675.r64932>

© 2020 Pereira M. This is an open access peer review report distributed under the terms of the [Creative Commons Attribution License](#), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

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 are an exsiccate number of this plant? Botanical Identification?

2. How much seeds and of powdered 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.

Reviewer Report 09 June 2020

<https://doi.org/10.5256/f1000research.18675.r47250>

© 2020 Sannanigaiah D. This is an open access peer review report distributed under the terms of the [Creative Commons Attribution License](#), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Devaraja Sannanigaiah

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.

The benefits of publishing with F1000Research:

- Your article is published within days, with no editorial bias
- You can publish traditional articles, null/negative results, case reports, data notes and more
- The peer review process is transparent and collaborative
- Your article is indexed in PubMed after passing peer review
- Dedicated customer support at every stage

For pre-submission enquiries, contact research@f1000.com

F1000Research