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Investigation of antimicrobial and anticoagulant effects of trypsin inhibitor from Caesalpinia ferrea var. cearensis

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***Abstract*— The species belonging to the Fabaceae family are abundant in the Brazilian region. They have great relevance because they are a source of plant proteins, and for its diversity of biomolecules with industrial potential and pharmaceutical applications. Among these are inhibitors, which can act in the regulation of endogenous proteinases and in the defense of plants against the attack of insects and microorganisms. This study reports the purification steps, partial characterization, in vitro effects of CfTI against pathogenic microorganisms, and hemostasis tests. *Caesalpinia ferrea* Trypsin Inhibitor (CfTI) was purified and partially characterized by standardized protocols for other inhibitors. Total protein was determined according to Bradford and the values were 4.7 mg/mL and 1.4 mg/mL in the crude extract and fraction eluted from affinity chromatography, respectively. CfTI reduced 96% on trypsin activity at 0.25 µg but did not inhibit chymotrypsin. Additionally, the inhibitor kept 85% of its activity up to 60 °C and about 90% in pH from 2 to 9. The electrophoresis on SDS-PAGE revealed only one band with molecular mass of approximately 18 kDa. CfTI prolonged aPTT up to 2.45 times, suggesting in vitro anticoagulant effect. CfTI was also tested with strain of pathogenic microorganisms, including bacteria and yeast, however, there was no growth inhibition. The data suggests that CfTI belongs to the Kunitz family with potential anticoagulant effect.**

Keywords: kunitz inhibitor; trypsin; blood coagulation

I. INTRODUCTION

The family Fabaceae stands out for the high content of polysaccharides, antioxidant components, and proteins in the seeds [1]. Among the great diversity on this family, *Caesalpinia ferrea* var. *cearensis* is considered one of the most, economically, relevant of the legume group [2]. It is native from the Amazon region, with wide distribution on north and northeast of Brazil [3]. It can be useful in food, textile, paper, cosmetics, and pharmaceutical industries [4].

The medicinal properties are interesting, with reports showing that it has potential against inflammation, ulcers, pathogenic microorganisms, and the seeds extract has an inhibitor for the herpes simplex virus [5-8].

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C. ferrea is an important specie as a source of biomolecules, in which are included the proteinase inhibitors (PIs), that can be present in different tissues of the plant, abundant in the seeds, making 5-15% of the total proteins. Those molecules act as anti-metabolic, because they form stable complexes, inhibiting reversibly, through competition the target enzymes [9]–[11]. Those molecules are interesting for bacterial growth inhibition [12] and can interfere on physiological processes as the coagulation cascade [13]. The present work describes the partial purification and characterization of the serine proteinase inhibitor isolated from *C. ferrea* seeds and its activity against microorganisms and in vitro activity on blood coagulation.

II. MATERIALS AND METHODS

A. Inhibitor Purification

Mature seeds of *Caesalpinia ferrea* var. *cearensis* were milled to a fine powder, and submitted to agitation for 2h at room temperature with 0.1 M NaCl (10%, wt/ml). The suspension was clarified by centrifugation (5000 g, 20 min, at 4°C). The supernatant was used for inhibitor purification, after dialysis against distilled water for 48h. Total protein extract from previous step were precipitated with 80% (ml/ml) aqueous acetone. The acetone-dried powder was dissolved in 0.02 M Tris-HCl buffer, pH 8.0 and centrifuged (5000 g, 20 min at 4°C). The clear supernatant was applied to a DEAE Sephadex column (13x3 cm), balanced with 0.1 M Tris-HCl buffer, pH 8.0. Protein elution was performed with linear gradient (Tris-HCl buffer 0.1 M/ NaCl 0 – 1M, pH 8.0), and fractions of 3 mL were collected. The absorbance at 280 nm was used to follow protein elution.

Bovine trypsin was used as enzymatic pattern. The fractions with biological activity were united in a pool, and dialyzed. The fractions with trypsin inhibition was applied to a column with the trypsin-Sepharose 4B resin (5 cm x 1 cm), previously equilibrated with 0.1 M Tris-HCl buffer pH 8.0. The retained material was eluted with 0.5 M KCl pH 2.0 and 2 mL fractions were collected. Monitoring of the chromatographic profile was performed by spectrophotometry at 280 nm.

The total extracts and fractions resulting from the exchange chromatography steps had their protein concentrations estimated according to the method of [14], using BSA protein as standard and the estimated protein concentration (mg/mL) in A595 nm spectrophotometer.

B. Measurement of bovine trypsin and chymotrypsin inhibition

The preincubation mixture, for the final volume of 2 mL, was composed of 250 µl of 0.1 M Tris/HCl buffer pH 8.0, 50 µl of bovine trypsin and 5, 10, 15 and 20 µl of the total extract, diluted five times. Preincubation lasted 10 minutes at 37 ° C. After this time, 1 ml of 0.5 mM DL-benzoyl arginine-paranitroanilide (DL-BAPNA) chromogenic substrate was added and the incubation continued for another 30 minutes at 37 ° C. The reaction was stopped by the addition of 500 µl of 30% (v / v) acetic acid and the hydrolysis of the substrate by the enzyme was measured spectrophotometrically at 410 nm.

For chymotrypsin measurement, the residual activity of the enzyme was performed involving the formation of a stable acyl-enzyme and the release of p-nitrophenyl, measured spectrophotometrically, as described by [15] and [16]. The DL-benzoyl-tyrosine-paranitroanilide (BTPNA) chromogenic substrate was dissolved in Dimethyl Sulfoxide (DMSO) to reach a concentration of 0.09 M, which was the stock solution. From the stock solution, it was diluted in 0.1 M Tris-HCl + 0.02 M CaCl 2 in the ratio 80%: 20% for use in the assay.

The preincubation mixture for the final volume of 1370 µl consisted of 100 µl of chymotrypsin (0.05 mg of the enzyme dissolved in 1 mM HCl) and 20 µl of the total extract. The preincubation lasted 10 minutes at 37 °C. After the time described, 1000 µl of the BTPNA solution was added and, after 20 minutes of incubation, the reaction was stopped by the addition of 250 µl of 60% (v/v) acetic acid. Hydrolysis of the substrate by the enzymes was monitored spectrophotometrically at 410 nm. The inhibitory activity for both enzymes was calculated by determining the residual activity of chymotrypsin in the assay, according to [17].

C. Characterization of the inhibitor

To determine the stability of the inhibitor, buffers with a pH range of 2 to 10 were used as follows: 0.1M sodium citrate buffer (pH 2.0-5.0), 0.1M monobasic phosphate buffer (PH 6.0-8.0) and 0.1M sodium bicarbonate buffer (pH 9.0-10.0). The inhibitor was incubated with 50 µl of the buffers for one hour at 37°C. The pH of the samples was adjusted to 8.0 and the respective activities analyzed. The thermal stability of the inhibitor was analyzed by incubation at different temperature ranges. 50 µl samples were heated for 30 minutes at 36°C, 40°C, 50°C, 60°C, 70°C and 10°C and subsequently each sample of each treatment above was rapidly cooled and inhibitor activity was checked.

D. SDS- PAGE

For the stacking and separation gel the solution of 30% acrylamide and 0.8% acrylamide was used. Stacking gel was at the concentration of 5% in Tris-HCl 1M, pH 6.8 buffer, and the separation gel at 12.5% in Tris-HCl 1M, pH 8.8 buffer. Samples containing 20 µl of the purified inhibitor, under reducing conditions, were dissolved in 1 M Tris–HCl buffer, 20% SDS, 87% glycerol (wt/ml) and 0.2% bromophenol. Coomassie Brilliant Blue stained protein bands. Molecular mass markers with range from 10 to 260 kDa were used.

E. Antimicrobial activity

The tests were performed with fractions of non-diluted inhibitor and with fractions serially diluted: 1:10, 1:100 and 1:1000 that were filtered in membrane filters of 0.22µm. The strains used on the experiments are ATCC bacteria (*Escherichia coli* and *Staphylococcus aureus*) and from clinical isolates (*Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Streptococcus agalactiae*, *Enterococcus faecalis*), and yeast strains of *Candida albicans* obtained from clinical isolates.

The antimicrobial activity was determined through the method of disc-diffusion in agar according to National Committee for Clinical Laboratory Standards (NCCLS). Petri dishes containing 20mL of agar Mueller-Hinton were seeded with 24h cultures from bacterial suspension with concentration of 1 to 2 x 108 CFU (0.5 on the scale of McFarland). Discs of sterile filter paper, diameter of 6mm, applied with 10µL of the sample were added to the dishes, and incubated in 37°C for 24h. The same procedure was made to determine the antifungal activity. As positive control, the antimicrobial ciprofloxacin was used (5µg/disc), and for negative control sterile distilled water. The evaluation of the antimicrobial activity was based on the measurement of the diameter of the inhibition halo formed around the disc.

F. aPTT and TP assays

Preparation of plasma pool

The plasma was obtained from the research team members, all adults and healthy, free from anticoagulants for at least two weeks, and after eight hours fasting. For this purpose, 4.5 ml of blood was collected in test tubes containing sodium citrate (proportion 9:1 v/v, blood: anticoagulant)

Prothrombin Time (PT)

The extrinsic pathway action was analyzed through PT, as described in the literature. The tests and controls were performed in duplicate, using the commercial reagent kit PT CLOT (Bios Diagnostica, São Paulo, SP, Brasil). To prepare the tests (total extract and fractions), 90µl of plasma was used with 10µl of each sample. Those were then heated to 37°C, from 3 to 5 minutes in water bath. In a test tube, 200 µl of the reagent PT CLOT (Bios Diagnostica) was added and incubated in 37°C for 5 minutes. It was pipetted 100 µl of the sample and control to the tube containing the reagent, stopping simultaneously the stopwatch. The tube remained in water bath and slowly agitated. Before the predicted coagulation time, the tube was removed from the water bath and the time of clot formation registered. The plasma pool was used as negative control, and the plasma with heparin as positive control. After this, the average was taken from the duplicated results.

Activated partial thromboplastin time (aPTT)

The intrinsic and common pathway were analyzed through aPTT, as described in the literature. The tests and controls were performed in duplicate, using the commercial reagent kit PT CLOT (Bios Diagnostica, São Paulo, SP, Brasil). To prepare the tests (total extract and fractions), 90µl of plasma was used with 10µl of each sample. Those were then heated to 37°C for 2 minutes in water bath, after 100 µl of aPTT CLOT reagent was added and incubated in 37°C from 2 to 3 minutes. Following, it was pipetted 100 µl of calcium chloride ($CaCl_2$ pre-heated), stopping simultaneously the stopwatch. With the tube on the water bath, it was slowly agitated. The tube was removed before the predicted time for coagulation, and the time for clot formation registered. The pool plasma was used as negative control, and plasma with heparin as positive control. After this, the average was taken from the duplicated results.

III. Results and Discussion

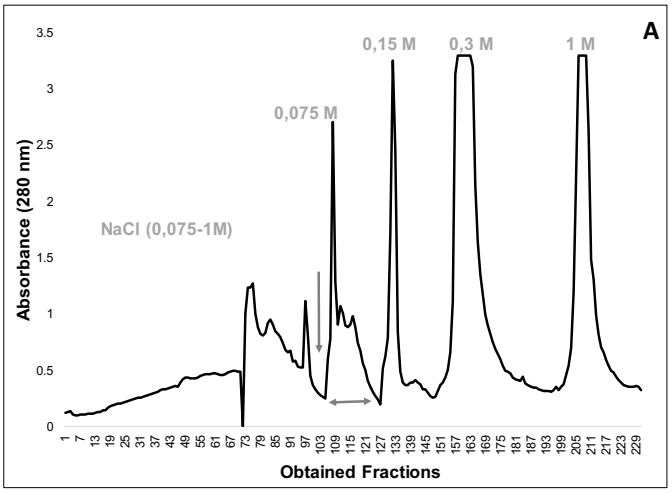
A. Detection of trypsin and chymotrypsin inhibition

The inhibitor extracted from *C. ferrea* var. *cearensis* seeds was active upon trypsin, however it did not inhibit chymotrypsin. For this reason, the inhibitor was named CfTI (*Caesalpinia ferrea* trypsin inhibitor). This difference may be attributed to the amino acid sequence on the reactive site of the inhibitor, where is necessary to inhibit trypsin the residues of Lys or Arg, while chymotrypsin has affinity for Phe, Tyr or Leu. It is possible that CfTI sequence does not have residues with affinity for chymotrypsin. Inhibitors with affinity for trypsin and have low or none affinity for chymotrypsin are the most common [18]–[21]. However, there are report of inhibitors that have affinity for both enzymes as the one extracted from *Acacia karroo*, with two binding sites that overlap, on position 68-69, the residues Arg-Ile bind to trypsin, and the residue Tyr right before Arg, binds to chymotrypsin [22].

B. Protein quantification

Proteinase inhibitors are purified and characterized from a variety of plant species [23], [22], [24]. The methods applied to extract proteins from *C. ferrea* var *cearensis* seeds, are commonly used for isolation of plant proteins. Among those, the extraction through water, saline and/or acids solutions, and precipitation by organic solvents or by salts, have been used to obtain proteolytic inhibitors [25]– [27].

The acetone extract from *C. ferrea* seeds had high concentration of total proteins (4.7 mg/ml), if compared for example with extracts of *Poincianella pyramidalis*, 1.36 g/mL [21]. After the ion exchange chromatography, the protein content of the fraction with inhibitory activity, that is the fractions eluted with 75 mM NaCl, presented concentration of 1.4 mg/ml. The variation of the protein content may be due to the extraction or solubility of proteins contained in seeds from different lineages [28].



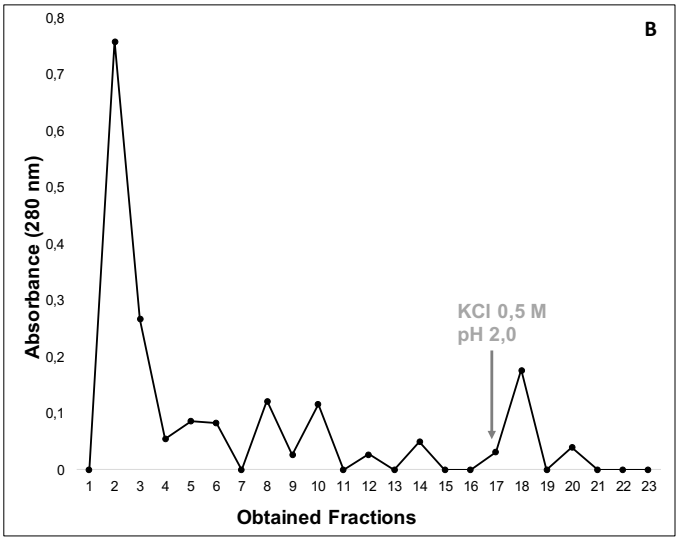


Fig. 1.**A** – Ionic exchange chromatography from the acetone precipitate obtained from *C. ferrea* var *cearensis* seeds. Balance buffer: Tris-HCl 0.1 M pH 8.0. Elution buffer: Tris-HCl 0.1M/NaCl (0.075 M – 1M). The elution was photometrically monitored in A280nm. Fraction (F1) with inhibitory activity. **1.B** Elution profile of the affinity chromatography in Trypsin-Sepharose. Balance buffer: Tris-HCl 0.1M pH 8.0. Elution buffer: KCl 0.5 M. The fractions were photometrically monitored in A280nm. CfTI indicated on fraction 18.

C. Ionic exchange chromatography

On Fig. 1A it is represented the chromatography profile from the acetone precipitate of *C. ferrea* var. *cearensis.*

From the obtained fractions, only the one eluted with 75 mM NaCl, here referred as F1, had inhibitory activity and it is where CfTI could be found. There is a main peak and two small ones on F1, that indicated the need for additional chromatography for the trypsin inhibitor purification. As in other studies [18], [29], the affinity chromatography in trypsin-Sepharose Fig. 1B was made to purify the inhibitor.

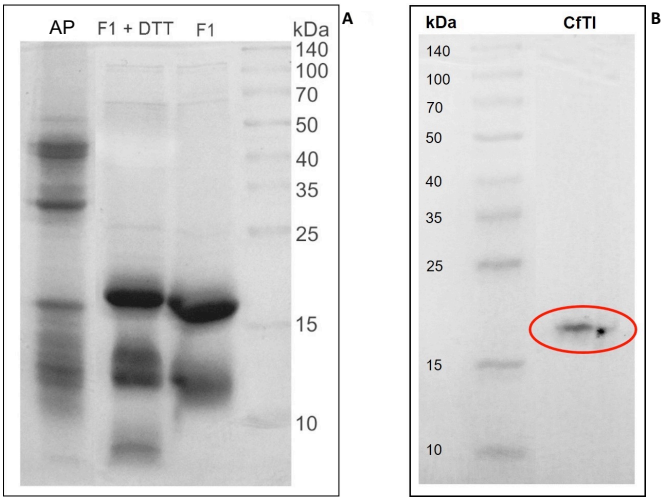


Fig. 2.**A** – Electrophoresis in SDS-PAGE of the obtained fraction from ionic exchange chromatography, DEAE-Sepharose. AP stands for acetone precipitate; F1+ DTT, F1 with addition of DTT; F1 (38,19 µg), the fraction with inhibitory activity without DTT. **2.B** Electrophoresis of fraction 18, obtained from affinity chromatography, first column – molecular markers, second column shows the fraction 18 (76,39 µg CfTI).

D. Affinity chromatography on trypsin-Sepharose

The affinity chromatography was done for CfTI purification, considering the 3 protein bands on polyacrylamide gel after ionic exchange chromatography Fig. 2A. After this step, the fraction 18 was obtained, which migrated as a single protein band Fig. 2B, when compared to the previous chromatographic step. The molecular mass was approximately 18 kDa, confirming the purification of the inhibitor. Reference [19] also purified a serine proteinase inhibitor from *Acacia schweinfurthii* (ASTI) seeds using the same chromatographic methods used to purify CfTI. However, the electrophoretic profile of ASTI showed that this inhibitor presents two protein bands in the presence of DTT, a reducing agent. However, the electrophoretic pattern of the F1 fraction, containing CfTI, did not differ after treatment with DTT Fig. 2A, justifying non-treatment with DTT in the fraction eluted from affinity chromatography Fig. 2B.

E. Influence of temperature and pH on inhibitory activity

The CfTI activity was evaluated in different temperatures (from 36 to 100°C), the inhibitor was stable until 60 °C with 85% of activity upon trypsin. When tested on 70°C, the activity dropped to 43%, and completely null when exposed to 100°C, Fig. 3A. On tests with different pH values, there was little variation on the inhibitory activity, on pH range from 2-9, with values around 90-95% with the reduction of inhibition on pH 10, Fig. 3B. These results are similar to a Kunitz type inhibitor isolated by [21], with high stability in different temperatures and pH.

F. Inhibition curve of bovine trypsin

For the inhibition essays, bovine trypsin active concentration was 2.89 mg/mL, and the acetone extract and fraction F1 were used to detect inhibition. From the

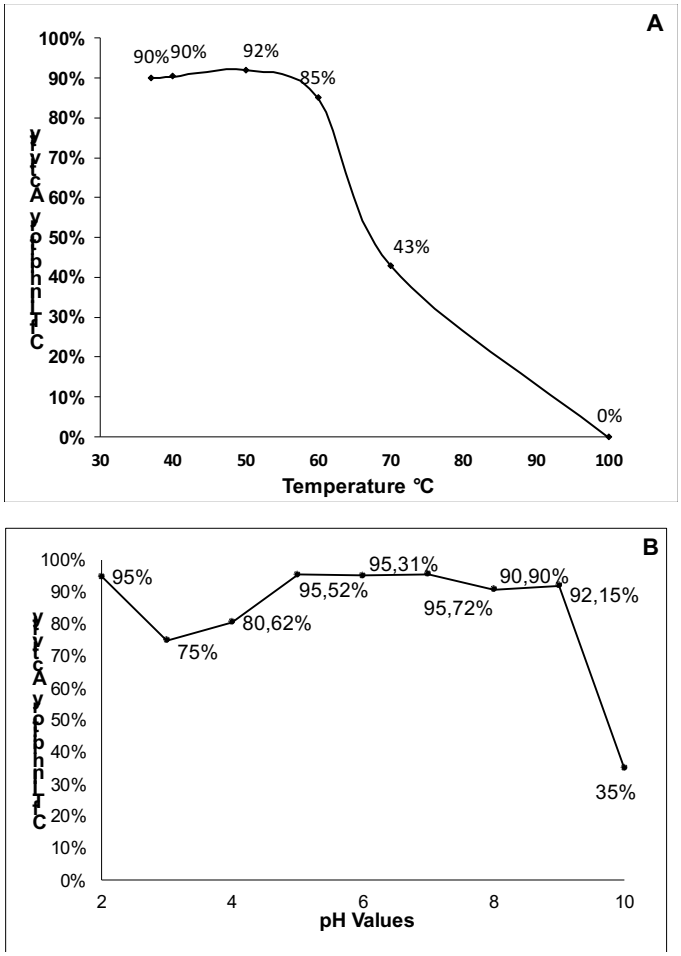


Fig. 3A. CfTI inhibitory activity in different temperatures, 36 to 100°C. **3B** different pH values from 2 to 10.

extrapolation of the inhibition curves to the point of intersection with abscissa, the concentration of inhibitor that promotes the reduction of residual trypsin activity can be calculated. The reduction in enzyme activity (25%) was obtained in the presence of approximately 0.2 µg of acetone extract (data not shown). Reference [30] isolated a Kunitz-type trypsin inhibitor of the species *Passiflora edulis* (Sims) and reported that with 0.5 µg of the partially purified inhibitor, trypsin activity was reduced by about 25%.

The inhibition curve using F1 (CfTI) shows that was a reduction of 50% of trypsin activity with 0.12 µg of the inhibitor and for only 4% of the residual enzyme activity with 0.25 µg, Fig. 4. This result, in comparison with the acetone extract, shows higher efficiency of F1 indicating higher activity of the inhibitor. In fact, the purified inhibitor may present less yield, however, may have higher specificity, evidencing the activity potential of the molecule.

Another important serine proteinase, chymotrypsin, was tested but was not inhibited by CfTI. In general, the inhibitors can fit into two categories: first, the ones that have two polypeptide chains and inhibit both trypsin and chymotrypsin, as the ones present in the subfamily Mimosoideae; the second group, present on the subfamily Caesalpinioideae, that has as

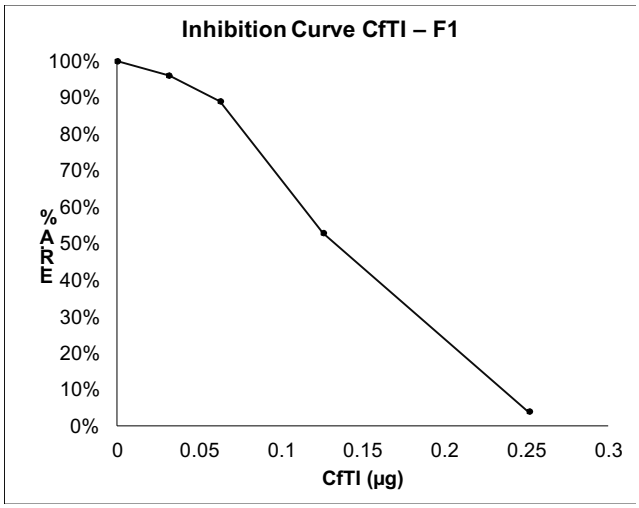


Fig. 4 – Enzyme residual activity (E.R.A) of trypsin in the presence of CfTI (F1). The inhibitory activity of trypsin active site was determined through essay in incubation with BAPNA in presence of different inhibitor concentrations.

only one polypeptide chain with low or none affinity for chymotrypsin and strong inhibition for trypsin [18], [19].

Other inhibitors described by [20], [21] from species as *Cedrelinga catenaeformis*, *Parkia pendula*, *Ormosia paraensis* and *Poincianella pyramidalis*, also presented strong affinity for trypsin and low or none for chymotrypsin.

G. Antimicrobial activity

According to the observation period for the incubated dishes, there was no inhibition on growth of the strains from the tested microorganisms in any of the tested samples (non-diluted fractions of the inhibitor and the serially diluted). The positive control (ciprofloxacin) inhibited as expected, and the negative control (sterile distilled water) did not interfere on strains growth.

H. Anticoagulant tests

The anticoagulant activity of CfTI fraction was evaluated through prothrombin time (PT) and activated partial thromboplastin time (aPTT), using normal citrated human plasma, as observed, CfTI extended the coagulation time on aPTT tests up to 2.45 times, demonstrating its anticoagulant activity in the intrinsic and/or the common coagulation pathway with discrete activity on the extrinsic pathway. Heparin was used as positive control, and as expected, showed significant anticoagulant activity with PT higher than 18 seconds (negative control: 13.11 ± 0.41 s) and aPTT higher than 117.3 s (negative control: 35.2 ± 4.0 s). As described by [31], non-fractionated heparin shows an irregular pharmacokinetics, and a strict therapeutic window, the usage of this drug requires constant monitoring to obtain appropriate dosage. The aPTT test is indicated for control of the intrinsic pathway, and the common pathway factors (X and IX), being sensitive to heparin with variation of 0.1 to 1 U/ml. There is a variation between individuals on the plasma concentration of heparin and on the anticoagulant effect in healthy young adults, as shown by [32]. The reason for such variation is not completely elucidated, but is believed that might be related in parts to the difference on the levels of coagulation factors [33], the differences on regulatory mechanisms and variation on the elimination of heparin [34]. The obtained results demonstrate the anticoagulant activity of CfTI, indicating the presence of bioactive compounds of pharmacologic interest. CfTI fraction showed up to be efficient in extending the aPTT in all patients, with no significant variation between individuals, as it happens with heparin. Further studies are necessary for identification and purification of such compounds, that in future might be used as an efficient and safe anticoagulant treatment. According to [35], protease inhibitors from plants potently inhibit the growth of a variety of pathogenic bacterial and fungal strains and are therefore excellent candidates for use as lead compounds for the development of novel antimicrobial agents. However, in this study CfTI did not show activity against pathogenic bacteria neither *Candida albicans*.

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