International Journal of Biological Macromolecules 148 (2020) 869–879

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

journal homepage: http://www.elsevier.com/locate/ijbiomac

GdTI, the first thermostable trypsin inhibitor from *Geoffroea decorticans* seeds. A novel natural drug with potential application in biomedicine

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ARTICLE INFO

Article history:
Received 2 December 2019
Received in revised form 15 January 2020
Accepted 21 January 2020
Available online 22 January 2020

Keywords:
Geoffroea decorticans
Trypsin inhibitor
Serine protease inhibitor
Anticoagulant activity
Antidiabetic drug

ABSTRACT

A novel thermostable trypsin inhibitor was obtained from *Geoffroea decorticans* seeds. *G. decorticans* trypsin inhibitor (GdTI) is a protein with molecular mass of 6743.7 Da, with a potent inhibitory activity (*Ki* of 2.1 nM) even at high temperatures and extreme pHs (100% after 5 h at 100 °C and 80% after 60 min at pH 2–12) constituting one of the most powerful serine protease inhibitors isolated from a plant source. GdTI displays anticoagulant activity against both extrinsic and intrinsic coagulation pathways, representing the first report of a plant serine protease inhibitor with anticoagulant activity against the extrinsic pathway. Finally, GdTI showed inhibitory activity against α-glucosidase (IC_{50} of 0.18 μM) evidencing the hypoglycemic effect of this inhibitor. Our results evidence the discovery of a natural molecule with unique features: i) GdTI is one of the most potent serine protease inhibitors founded to date, ii) with the most powerful thermostability reported in literature, iii) with anticoagulant effect against both coagulation pathways and hypoglycemic activity. This report suggest that GdTI could be exploited as a natural and hyperstable antidiabetic drug, in behalf of its antithrombotic and hypoglycemic activities, encouraging future studies with high impact on biomedical research and potential pharmaceutical applications.

1. Introduction

Peptide protease inhibitors (PPIs) are chemical compounds of low molecular weight found in tissues and fluids of animals, plants and microorganisms. Some of its main functions include to prevent the biological action of proteases and preclude the action of pathogenic organisms [1]. PPIs have important technological applications in biomedicine, biotechnology, food industry and diagnosis [2,3]. It is known that the natural products constitute one of the major sources of raw material for the development of innovative medicine and antimicrobial agents. The obtaining process of these natural products involves procedures relatively simple and with a minor cost than those obtained by chemical synthesis. In particular, the plant sources of PPIs are a field sparsely explored, even though its show an extraordinary wealth and diversity. For that reason and considering that the botanical material of the Latin-America is yet little explored, the study of new PPIs of natural sources capable of modifying (or modulating) the biological activity of proteases becomes relevant. An example of this is *Geoffroea decorticans* (Gill. ex Hook. et Arn.) Burkart (Fabaceae); a xerophilous deciduous tree present in most arid forests of southern South America (Chile, Argentina, Bolivia, Chile, Paraguay, Peru and Uruguay) and whose popular name is chañar. Its leaves, bark, flowers and fruits have been studied, demonstrating the presence of chemical components with specific properties. Based on some studies in the literature, compounds with antifungal properties have been found in the organic extracts of the chañar stem bark, so this plant could be expected to be useful for the treatment of pain, especially associated with inflammatory processes [4–7]. In addition, their fruits have been consumed by humans in rural communities since ancient times [8,9]. The seeds of chañar are well known and have been previously investigated for its chemical composition and nutritive properties: they constitute a good source of essential nutrients, such as proteins, carbohydrates and lipids [8,10,11]. Despite all these studies, there not have been previously reported any investigations demonstrating the presence of protease inhibitors in the seeds of chañar. After an extensive literature search, only one mention could be found in relation to trypsin inhibitory activity on the crude extract of chañar by Becker [12] who studied the nutritional quality of the fruit from the chañar tree, without any isolation or purification of the potential trypsin inhibitor.

The potentiality and therapeutic efficacy of PPIs have been reported for different diseases: immune, inflammatory and respiratory diseases [13], cardiovascular and neurodegenerative diseases (such as Alzheimer's disease) [14], AIDS [15], hepatitis [16], cancer [17,18] and malaria [19],

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https://doi.org/10.1016/j.ijbiomac.2020.01.214
0141-8130/

among others [20]. Despite all those medical applications reported, the use of PPIs as inhibitors of enzymes involved in metabolic disorders has been little studied. Therefore, the search for new alternative agents of natural origin that could be applied in such pathologies becomes relevant. For example, various therapeutic alternatives have been applied in hyperglycemia disorders, with the main purpose of reducing postprandial hyperglycemia and reducing the microvascular complications associated with the deregulation of blood glucose levels [21]. In this context, the inhibition of the enzyme α-glucosidase—responsible for catalysing the release of glucose from complex carbohydrates—has been effective in reducing blood glucose levels [22]. An example of these diseases is Diabetes mellitus, which is characterized by hyperglycemia in association with impaired carbohydrate, lipid and protein metabolism [23]. Current treatments that involve the use of drugs with hypoglycemic activity—such as acarbose, voglibose and miglitol—although they are effective, have serious side effects at the gastrointestinal level. For its part, it is known that thrombotic events due to blood coagulation represent a serious problem in cardiovascular diseases [24–26]. Although heparin has been used for this purpose in the last 50 years, its use results in the development of thrombocytopenia and immune response when used for a long time [27]. In addition, other anticoagulant drugs such as aspirin and clopidogrel result in serious side effects [28], so that the finding of new PPIs with inhibitory activity of the coagulation cascade would result in an attractive strategy against thrombosis. Under this context, the search for new anticoagulant and hypoglycemic agents from natural origin is of great interest and relevance given their potential use and implication in biomedical therapies related to these metabolic diseases.

The discovery of new molecules from natural sources and with biological activities, low molecular weight, high physicochemical stability, low toxicity and ease of metabolism in the human body, offers wide advantages over synthetic compounds, allowing their utilization without special storage and/or administration conditions (such as low temperature storage, addition of stabilizing agents, preservation in buffers, among others). In particular, in the biomedical field, the majority of actually used agents for the treatment of diverse diseases require severe storage conditions. Many of those agents are very labile, having little stability against shifts in pH and/or temperature; while others are macromolecules of high molecular weight, which feature hinders their access to regions of fine vascularization and makes their half-life usually quite short. The availability of stable natural molecules with relevant biological activities fulfill the criteria for industrial use in the form of a profitable, ecologically friendly, and versatile product that is suitable for a wide geographical distribution with respect to commercialization and use in multiple areas with different climate conditions.

PPIs are classified in six classes according to the type of protease with which they interact, namely: serine, cysteine, aspartic, metallo, glutamic and threonine protease [2,29]. Most of the plant PPIs are serine protease inhibitors [30] and as such are classified into >20 families. In general, plant PPIs of this family are small proteins (molecular weight *ca.* 4–8 kDa) with several cysteine residues forming intrachain disulfide bonds or interstrand disulfide bridges, exhibiting a compact and stable structure that usually confers physicochemical stabilities such as resistance to high temperatures, extreme pHs, and/or high salinity, among others [31]. Several studies carried out in recent years have demonstrated that the high physicochemical stability of PPIs is usually a consequence of their structure [32–36]. Additionally, studies focussed on the characterization of plant PPIs have demonstrated that those molecules not only exhibited the aforementioned conditions—*i. e.*, low molecular weight, physicochemical stability, and natural origin—but also a wide range of biologic activities. Several studies have been achieved in serine protease inhibitors demonstrating its potential use in pathologies such as cancer [17,18], dermatitis [20], cardiovascular diseases [37,38], AIDS [39] and inflammation [40], where trypsin activity is deregulated. These features have stimulated the investigation of plant PPIs as promising candidates for potential application in biomedicine, agriculture, and/or the food industry.

Motivated by the need to find new plant species that can represent novel sources of proteins, this work was aimed at evaluating the chañar seeds as new sources of protease inhibitors. In this study, we present the isolation and purification of, as far as we understand, the first thermostable trypsin inhibitor of *G. decorticans* (GdTI), with remarkable physicochemical stability and one of the most potent inhibitors isolated to time, focusing on its potential as a natural antithrombotic and antidiabetic drug.

2. Material and methods

2.1. Materials and reagents

The ripe fruits of *G. decorticans* (chañar) were hand-collected from trees located in the department of Juana Koslay (33°16′02.9″S 66°12′50.2″W), in the province of San Luis, Argentina, in the course of January 2018. Coomassie Blue G-250, *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED), sodium chloride, tris (hydroxymethyl) aminomethane, sodium dodecyl sulphate (SDS), β-mercaptoethanol (βME), bovine serum albumin (BSA), Nα-benzoyl-DL-arginine-p-nitroanilide (BApNA), Trypsin, 4-nitrophenol-α-D-glucopyranoside (PNPG) and α-glucosidase were purchased from Sigma-Aldrich (U.S.A.). Glyoxyl-agarose was delivered from FlukaTM. All other chemicals used in this work were of analytical grade.

2.2. Crude extract preparation

The woody endocarp of *G. decorticans* (chañar) fruits was hand-broken in order to obtain the chañar seeds. Approximately 10 g of seeds were washed with distilled water and grounded using a blender with addition of 100 mL of 0.1 M Tris-HCl buffer, pH 7.5 on ice bath to avoid possible protein denaturation. After incubation for 120 min at 4 °C, the mixture was filtered through a fine screen cloth and centrifuged for 30 min at 7000 ×*g* at 4 °C. The clarified supernatant (from now on: GdCE) was collected and immediately frozen at −20 °C until analysis. The protein content and trypsin inhibitory activity were determined as described below.

2.3. Determination of total protein content

The total protein content was determined by the Bradford's assay, as described [41] using bovine serum albumin (BSA) as standard (0–1 mg mL^{-1}). 10 μL of each standard solution or test sample were mixed with 200 μL of the Bradford reagent and after 10 min incubation at room temperature, the absorbance at 595 nm was measured (Tecan Infinite M200 PRO, Männedorf, Switzerland). When the dye is linked to the protein, the maximum absorbance is shifted from 465 nm (free dye) to 595 nm (dye-protein). This method results especially suitable for the determination of protein content of vegetable extracts, which usually presents phenolic compounds that interferes with the traditional Lowry method. Measurements were carried out in triplicate.

2.4. Trypsin inhibition measurements

Trypsin inhibitory activity was determined by using the substrate Nα-benzoyl-DL-arginine-p-nitroanilide (BApNA) according to Erlanger, Kokowsky & Cohen [42] with slight modifications and adapted to a 96-well plate. A fixed amount of trypsin (0.25 mg mL^{-1}) was pre-incubated with different concentrations of the extract (ranging from 0 to 50 μg mL^{-1}) in 0.1 M Tris-HCl buffer (pH 7.5) containing 50 mM $CaCl_2$. After 10 min pre-incubation at 37 °C, the substrate was added to each reaction mixture at a final concentration of 1 mM. The hydrolysis of BApNA was recorded through the increase of the absorbance at 410 nm at 37 °C every minute for 10 min. One trypsin inhibitory unit (1 TIU) was defined as the decrease in 0.01 unit of absorbance at 410 nm per 10 min assay, at 37 °C. Measurements were carried out in triplicate and appropriate blanks were also included.

Table 1
Concentration and IC_{50} values for GdCE, GdHT40-100[a].

Sample	Concentration (μg mL^{-1})	IC_{50} (μg mL^{-1})
GdCE	171.2 ± 8.7	4.28 ± 0,21
GdHT40	85.8 ± 6.3	2.15 ± 0.15
GdHT50	51.2 ± 3.5	1.28 ± 0.18
GdHT60	47.1 ± 4.7	1.17 ± 0.23
GdHT70	44.2 ± 1.2	1.11 ± 0.15
GdHT80	45.3 ± 5.2	1.13 ± 0.13
GdHT90	26.2 ± 9.6	0.66 ± 0.17
GdHT100	22.3 ± 7.4	0.55 ± 0.18

[a] GdCE: *Geoffroea decorticans* crude extract, GdHT40–100: *Geoffroea decorticans* crude extract after 40–100 °C heat treatment for 30 min.

2.5. Purification

2.5.1. Partial purification by heat treatment

The crude extract (GdCE) was subjected over a wide temperature range (40 °C, 50 °C, 60 °C, 70 °C, 80 °C, 90 °C and 100 °C) on water bath for 30 min. After cooling at room temperature, thermally denatured proteins were removed by centrifugation for 30 min at 7000 ×*g* and 4 °C. Afterwards, the total protein content and the inhibitory activity of the non-treated crude extract and heat-treated samples were determined. Each obtained sample was called GdHT40, GdHT50, GdHT60, GdHT70, GdHT80, GdHT90 and GdHT100, in accordance with the incubation temperature used.

2.5.2. Molecular exclusion chromatography

A 2 mL aliquot of GdHT90 (80 μg mL^{-1}) was loaded onto a Sephacryl-S100 HR column (1.5 × 40 cm) connected to an Äkta-Purifier (GE Healthcare) previously equilibrated with 0.1 M Tris-HCl buffer (pH 7.5). Elution was carried out at a flow rate of 0.8 mL min^{-1} monitoring 280 nm absorbance, and fractions were evaluated for trypsin inhibitory activity as described before.

2.5.3. Affinity chromatography purification

All the fractions that presented trypsin inhibitory activity were pooled and loaded onto a trypsin-glyoxyl-agarose column prepared in house following the method of Obregón and colleagues [43] (1.5 × 12 cm) connected to an Äkta-Purifier (GE Healthcare) previously equilibrated with 0.1 M Tris-HCl buffer (pH 7.5). After complete removal of the non-retained proteins with equilibration buffer, affiliated proteins were eluted generating a linear pH gradient with Glycine-HCl buffer (pH 2.6) at a flow rate of 0.7 mL min^{-1}. The eluted fractions were adjusted to pH 7.0 by adding NaOH. The purified trypsin inhibitor was named GdTI (*G. decorticans* Trypsin Inhibitor). Trypsin inhibitory activity and protein quantification were determined as previously described.

2.6. Characterization of GdTI

2.6.1. SDS-PAGE analysis

GdCE and GdHT (GdHT40-GdHT100) were analysed by SDS-PAGE in order to determine the purity of the protease inhibitor during the different purification steps. Samples were prepared in sample buffer (Tris 0.13 M, SDS 2%, β-mercaptoethanol 5% *v*/v, glycerol 8% v/v, bromophenol blue 0.002% *w*/*v*, pH 6.8) and incubated for 5 min at 100 °C, then subjected to denaturing electrophoresis at constant current of 15 mA per gel using a Mini-Protean III electrophoretic cube (Bio-Rad, Hercules, CA 94547, USA), using a 4% T, 3% C stacking gel and a 12% T, 3% C separating gel. Gels were silver stained following the Chevallet, Luche & Rabilloud [44] protocol.

2.6.2. Molecular mass determination by MALDI-TOF/MS

Mass Spectrometry analysis were performed at the Proteomics Core Facility CEQUIBIEM, at the University of Buenos Aires/CONICET (National Research Council) as follows: the sample was mixed with an equal volume of 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid) matrix solution. Previously, the sample was desalted by dialysis in 0.022 μm filters. The matrix-sample mixture was spotted onto MTP 384 target plate polished steel TF from Bruker Daltonics, and the spots were evaporated to dryness at room temperature. Mass spectra were acquired on a Bruker Daltonics Ultraflex MALDI-TOF mass spectrometer (Bruker Daltonics, Billerica, MA, USA) operating in a linear positive mode.

Peptide identification was achieved by tryptic digestion and posterior analysis by nano HPLC coupled to a mass spectrometer with Orbitrap technology. The analysis of the data obtained was performed with the Proteome Discoverer 1.4 program (Thermo Scientific) using the *Fabaceae* database. Identified peptides were aligned by BLASTP software.

2.6.3. Effect of temperature and pH

For thermal stability assay, GdTI aliquots were incubated at 40 °C, 50 °C, 60 °C, 70 °C, 80 °C, 90 °C and 100 °C for 1 h and then cooled at room temperature. Temperature stability was also determined for a fixed temperature (90 °C and 100 °C) for 0.5, 1, 1.5, 2, 2.5, 3, 4 and 5 h. In both cases, the residual trypsin inhibitory activity of GdTI was determined using BApNA as substrate as previously described in Section 2.4.

The effect of pH on GdTI stability was evaluated by measuring the residual activity after incubation at various pH values (pH 2, pH 4, pH 6, pH 8, pH 10 and pH 12) for 1 h at 25 °C. The residual trypsin inhibitory activity of GdTI was determined as previously described in Section 2.4, using BApNA as substrate.

2.6.4. IC_{50} value and kinetic studies

The amount of GdTI needed for 50% inhibition of trypsin activity was determined using different concentrations of the inhibitor. To calculate both the inhibition constant (*Ki*) of GdTI, the Dixon plot (1/v *vs* [I], where [I] is the inhibitor concentration) was used. Assays were performed using increasing GdTI (0–3.2 μg mL^{-1}) and BApNA (1.0 and 2.0 mM) concentrations for the kinetics of trypsin inhibition. Bovine trypsin was used at 0.25 mg mL^{-1} fixed concentration and reactions were performed as previously described. The reciprocal of the enzyme reaction rate was expressed as 1/v, and *Ki* value was calculated from

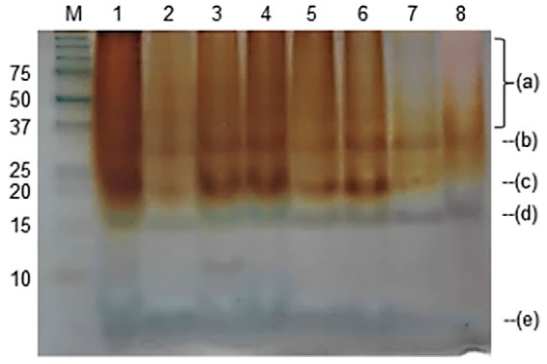

Fig. 1. Electrophoresis (SDS-PAGE, 12% *v*/v) of the protein fractions obtained after partial purification by heat treatment: lane M, molecular mass marker; lane 1, crude extract of *Geoffroea decorticans* seeds (GdCE); lane 2, GdCE after 30 min treatment at 40 °C (GdHT40); lane 3, GdCE after 30 min treatment at 50 °C (GdHT50); lane 4, GdCE after 30 min treatment at 60 °C (GdHT60); lane 5, GdCE after 30 min treatment at 70 °C (GdHT70); lane 6, GdCE after 30 min treatment at 80 °C (GdHT80); lane 7, GdCE after 30 min treatment at 90 °C (GdHT90); lane 8, GdCE after 30 min treatment at 100 °C (GdHT100). Fractions (a), (b), (c), (d) and (e) indicate the protein fractions of approximately 30 kDa, 20 kDa, 15 kDa and <10 kDa respectively.

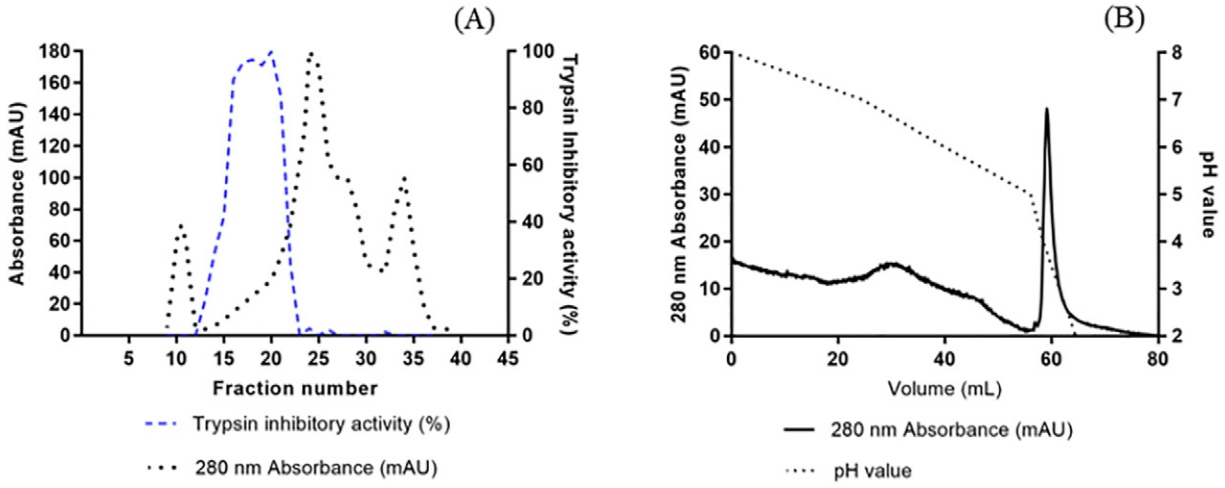

Fig. 2. Purification of *Geoffroea decorticans* trypsin inhibitor by size exclusion chromatography (Panel A) and posterior affinity chromatography on immobilized trypsin (Panel B).

the intersection of the two lines plotted for two different BApNA concentrations in the Dixon plot.

2.7. Biological assays

2.7.1. Anticoagulant activity

Anticoagulant activity of GdTI was evaluated by determining prothrombin time (PT) and the time of activated partial thromboplastin (aPTT) using a Coatron M1 coagulometer (TECO, Germany). In both cases, a pool of blood plasmas from the mixture, in equal parts, of 5 healthy individuals, maintained at 37 °C with 3.8% sodium citrate (ratio sample:anticoagulant 9:1) was used as a sample (from now on: PBP).

For the PT test the commercial Soluplastin reactive (Wiener Lab.) was employed. Initially, equal parts of the PBP sample and the trypsin inhibitor (0–180 μg mL^{-1}) were incubated for 2 min at 37 °C, then 50 μL of Soluplastin were added to 25 μL of this mixture and checked for the coagulation time. For the aPTT test, 25 μL of aPTT (Wiener Lab.) were added to an equal volume of PBP-inhibitor mixture (previously incubated for 2 min at 37 °C). After 2 min incubation at 37 °C, 25 μL of 50 mM $CaCl_2$ were added to initiate the coagulation time determination. For both assays, measurements were carried out in triplicate and appropriate controls were achieved.

2.7.2. Hypoglycemic activity

α-Glucosidase inhibitory activity assay was performed according to Kim, Wang, & Rhee [45] with slight modifications and adapted to a 96-well plate, using the substrate 4-nitrophenol-α-D-glucopyranoside (PNPG). A fixed amount of α-glucosidase (0.2 U mL^{-1}) was pre-incubated with different concentrations of the inhibitor (ranging from 0 to 2.24 μg mL^{-1}) in 50 mM sodium phosphate buffer (pH 6.8). After 15 min pre-incubation at 37 °C, the substrate was added to each reaction mixture at a final concentration of 0.2 mM. The hydrolysis of PNPG was recorded through the increase of the absorbance at 405 nm at 37 °C every minute for 20 min. Measurements were carried out in triplicate and appropriate blanks were also included.

2.8. Statistical analysis

Statistical analyses (ANOVA) were performed with Graph Pad Prismv. 01 (http://www.graphpad.com/scientific-software/prism/). Significant differences between the means of the parameters were determined by Tukey's a-posteriori test ($p < 0.05$).

3. Results and discussion

3.1. Isolation and purification of the trypsin inhibitor from chañar seeds

Peptide protease inhibitors (PPIs) are molecules involved in the regulation of several biologic processes, by means of the prevention of unwanted proteolysis. At the present time, PPIs have a number of applications in biomedicine, biotechnology, the food industry, and diagnosis. Particularly, a wealth of increasing evidence has demonstrated that the plant PPIs have both preventive and therapeutic effects on several common cancers, as well as on multiple sclerosis, inflammatory processes, and a number of other diseases [46–49]. For this reason, with the main objective of amplify the pool of biological molecules with regulatory action that could be used in the biomedical field, we focused our study in the isolation and purification of a new protease inhibitor of vegetable origin, evaluating biologic activities that have been scarcely explored for this kind of molecules. Consequently, the seeds of *G. decorticans*, a tree regionally recognized for its use as a source of medicinal extracts with anti-inflammatory and antitussive properties [4,6,11,50] were considered as our study material. Although, both the nutritional content of the chañar seeds and the isolation of various phenolic compounds have already been described, either molecules of protein origin from this plant sample have not been reported to date. Accordingly, this study represents not only the first report of a protease

Table 2
Purification steps of GdTI from *Geoffroea decorticans* seeds.

Purification step	Total protein amount (mg)	Total Inhibitory activity, IA (TIU)[a]	Specific inhibitory activity, SIA (TIU mg^{-1})	Purity (fold)[b]	Yield (%)[c]
Crude extract	3.59 ± 0.27	21.35 ± 3.25	5.94 ± 0.46	1	100
90 °C heat treatment	1.2 ± 0.13	18.02 ± 2.47	15.02 ± 0.43	2.53	84.4
Size exclusion chromatography	0.72 ± 0.08	13.49 ± 0.68	18.74 ± 1.15	3.15	63.2
Affinity chromatography	0.11 ± 0.01	8.51 ± 0.23	75.98 ± 4.77	12.79	39.86

[a] One trypsin inhibitory unit (1 TIU) is defined as the decrease in 0.01 unit of absorbance at 410 nm per 10 min assay, at 37 °C.
[b] The purification index was calculated as the ratio between the specific inhibitory activity at each purification step and that of the crude extract taken as 1.
[c] Yield was calculated based on the total inhibitory activity recovered.

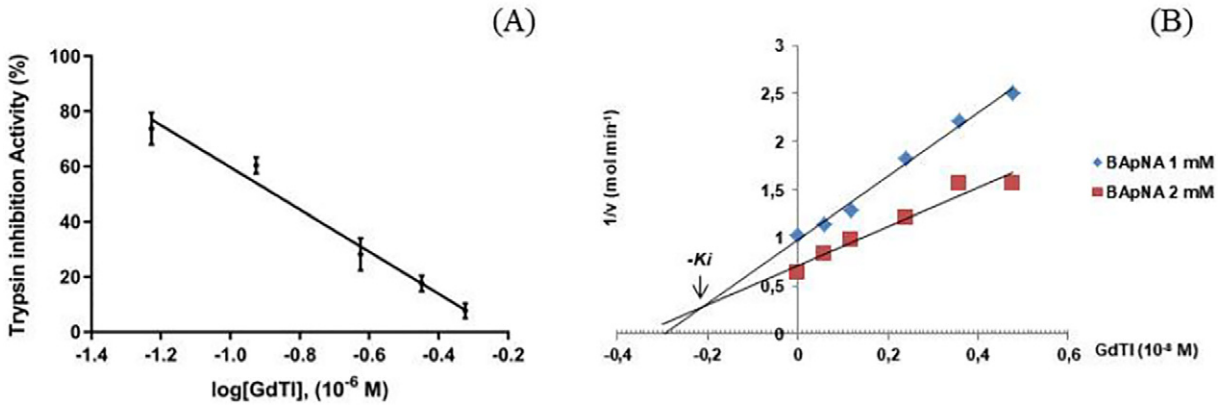

Fig. 3. Kinetic studies on GdTI trypsin inhibitory activity. (A) Dose response curve for IC_{50} determination. (B) Dixon plot (1/v *vs* [I]) for identification of *Ki* parameter. Each point represents the mean of three estimates.

inhibitor isolated from chañar seeds but also represents the first protein isolated, purified and characterized of said plant.

According to the methodology detailed in Section 2.2, a crude extract (GdCE) showing a total protein content of 171.2 ± 8.7 μg mL^{-1} and trypsin inhibitory activity of 21.35 ± 3.25 TIU (Section 2.4), was obtained.

3.1.1. Partial purification by heat treatment

Based on previous studies which evidence that PPIs present high physicochemical stability with minimal loss of inhibitory activity [32–36]—essentially attributed to the presence of numerous disulfide bridges relative to the total number of constituent amino acid [31]—, we proposed the treatment of GdCE at high temperatures for 30 min as initial purification step. We evaluate the residual trypsin inhibitory activity after submitting GdCE to 40, 50, 60, 70, 80, 90 and 100 °C for 30 min, with subsequent remotion of denatured proteins by centrifugation. As observed in Table 1, by means of this partial purification strategy it was possible to reduce the total protein content in the heat-treated fractions without significant trypsin inhibitory activity loss. Thus, an IC_{50} of 0.66 ± 0.17 μg mL^{-1} and an IC_{50} of 0.55 ± 0.18 μg mL^{-1} stand out for heat treatment at 90 °C and for the HT100, respectively. A general reduction behaviour in the IC_{50} value can be seen as a function of the increase in temperature, which is consistent with a partial purification of the trypsin inhibitor of chañar.

In order to evaluate the protein distribution in the different heat treatments, an assay of SDS-PAGE was carried. A progressive decrease in those bands corresponding to high molecular weight proteins can be observed (Fig. 1). Thus, a decrease in protein fractions with molecular weights higher than 30 kDa (fraction (a) in Fig. 1) as a function of the increase in temperature, was observed. This fact may indicate the removal of thermolabile proteins. In addition, 4 protein fractions of approximately 30 kDa, 20 kDa, 15 kDa and <10 kDa (fractions (a), (b), (c), (d), and (e) respectively in Fig. 1) are not affected by the temperature increase, which confirms the presence of thermostable peptides or proteins.

Considering that the heat treatment after 30 min incubation at 90 °C constitutes a simple and effective method for the removal of thermolabile proteins, it was selected as the initial sample to be loaded in the size exclusion chromatography column. Although the heat treatment at 100 °C ensures efficient protein removal, the IC_{50} values indicate that there are no significant differences between both treatments (see Table 1). For that reason, the heat treatment at 90 °C was selected to continue the purification steps considering that the sample is subjected to a minor thermal stress and, additionally, represents a purification step with minor operative expenses in case of a future industrial-level scaling.

3.1.2. Molecular exclusion and affinity chromatography purification

The second step for the purification of the PPI was to subject the GdHT90 to a molecular exclusion chromatography on a Sephacryl S-100 HR column (Section 2.5.2). The eluted fractions were monitored by absorbance at 280 nm and the trypsin inhibitory activity was determined in each of them (Fig. 2, panel A). Next, those fractions of the molecular exclusion chromatography that presented trypsin inhibitory activity were loaded on a column with immobilized trypsin in glyoxyl agarose (Section 2.5.3). After elution of non-retained proteins, an acidic pH gradient was applied that eluted the trypsin inhibitor, which we call GdTI (Fig. 2, panel B).

Under such selected conditions of work, the partial purification by 90 °C heat treatment for 30 min was effective for remotion of high molecular weight proteins, observing that the specific trypsin inhibitory activity increased 2.53-fold (Table 2). Subsequent purification steps (size exclusion chromatography and affinity purification) showed a specific inhibitory activity of 18.74 ± 1.15 TIU mg^{-1} protein (3.15-fold purification) and 75.98 ± 4.77 TIU mg^{-1} protein (12.79-fold purification) respectively, compared to the crude extract.

3.2. Characterization of the G. decorticans trypsin inhibitor (GdTI)

3.2.1. Inhibition kinetics

Kinetic studies of inhibition of trypsin by GdTI showed that the amount of inhibitor needed for 50% trypsin inhibition (IC_{50}) was 0.22 μM (Fig. 3, panel A), while *Ki* was on 2.1 nM (Fig. 3, panel B), indicating that inhibition of trypsin activity occurred at very low concentration of GdTI, characterizing a very potent inhibitor. Similar *Ki* values were reported for other serine protease inhibitors (Table 3). *Ki* values in the nanomolar range indicate a strong bond between the inhibitor and the target protease, requiring low concentrations of the former to produce a considerable reduction in enzyme activity. The importance

Table 3
Inhibitory constants of plan trypsin inhibitors.

Inhibitor name/s	*Ki* (nM)	Reference
Wild Soja trypsin inhibitor I	4.6	[51]
Cassia fistula trypsin inhibitor-1	2.9	[52]
Sapindus saponaria trypsin inhibitor	2.4	[53]
Entada acaciifolia trypsin inhibitor	1.75	[54]
Moringa oleifera protease inhibitor	1.5	[55]
Cratylia mollis trypsin inhibitor	1.4	[56]
Poincianella pyramidalis trypsin inhibitor	1.2	[57]
Butea monosperma protease inhibitor	1.2	[58]
Geoffroea decorticans trypsin inhibitor	2.1	

Ki: dissociation constant.

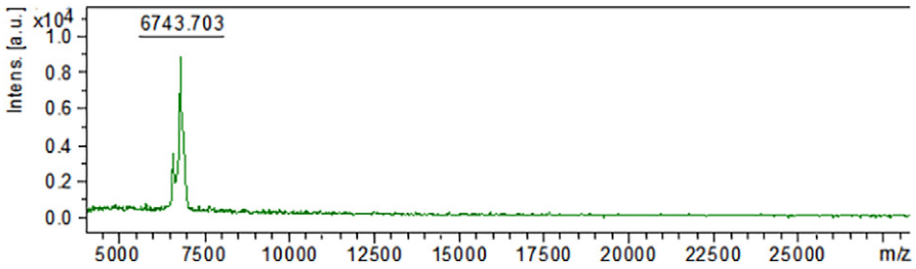

Fig. 4. MALDI-TOF mass spectrometric analysis of the purified *Geoffroea decorticans* trypsin inhibitor.

in the search of molecules that present this characteristic is based on the ability to exert a certain biological activity when the inhibitor is delivered in low doses, which strongly decreases the chances of producing unwanted side effects. In addition, by requiring small doses of inhibitor to perform its function, production costs for its isolation and purification are considerably reduced, which makes it more promising for industrial-level scaling.

3.2.2. Molecular mass determination by MALDI-TOF/MS

For molecular mass determination, GdTI was submitted to mass spectrometry analysis on a Bruker Daltonics mass spectrometer resulting on a 6743.7 Da signal (Fig. 4). However, the mass estimated by SDS-PAGE was approximately 20 kDa, tripling the value obtained by MALDI-TOF/MS (Supplementary Fig. 1). These results agree with other reports in which it was observed that many trypsin inhibitors have a tendency to self-associate, resulting in the formation of complex dimers, trimers and oligomers when they are in solution [59–67]. Although the reducing conditions of the SDS-PAGE would allow the visualization of the trypsin inhibitor monomers, other authors have reported the presence of bands corresponding to the different three-dimensional forms of the PPIs, concluding that the reduction and alkylation of disulfide bridges is not complete under these conditions [59,62]. Numerous studies have been carried out to elucidate the molecular structure of said inhibitors in an attempt to justify the mentioned behaviour. Finally, it has been reported that these inhibitors have a polar domain interface that is solvated with internal water molecules and hydrophobic patches that would be responsible for the tendency to self-association [61,68,69]. For all the above, we are able to affirm that the trypsin inhibitor of chañar has a molecular mass of 6743.7 Da with tendency to the formation of trimers in solution, even under the reducing conditions used in the SDS-PAGE.

GdTI was also submitted to tryptic digestion and posterior analysis by nano HPLC coupled to a mass spectrometer with Orbitrap technology, which allowed the separation of the obtained peptides and a posterior identification. The obtained spectra were analysed with the Proteome Discoverer 1.4 program, using the Fabaceae database. By this methodology it was possible to identificate a peptide with sequence SNPPQCR that matches with 63–69 residues of the Bowman-Birk trypsin inhibitor of *Glycine* max (Accession number RZB69152). Analysing this sequence we observe that it belongs to the conserved domain of Bowman-Birk inhibitors (BBI), which allows us to affirm that GdTI belongs to the BBI superfamily (Supplementary Fig. 2). Since we are in presence of the first isolated protein of chañar, a plant specie whose genome is not sequenced, it was not possible to identify other peptides, which accounts for the unique nature of the inhibitor studied here.

3.2.3. Temperature and pH stability

As mentioned earlier, protease inhibitors are recognized for their high physicochemical stability [32–36]. Several plant PPIs have been purified and found to be highly stable and quite active up to 70 °C [70]. Even PPIs stable in a wide temperature range have been found, maintaining their activity after incubating at temperatures close to 100 °C. Likewise, a pH stability study indicated that plant PPIs were functionally stable within different pH ranges, and even under highly acidic and alkaline conditions (pH 2–12). For this reason, PPIs are among the most stable proteins found in nature. For most biotechnological applications, highly thermostable proteins are necessary, in order to diminish the environmental impact of the industrial activity [71]. Thus, the discovery of new proteins with stability to extreme temperatures and pHs provides a useful tool for their application in a wide range of biotechnological processes, allowing a rational use of natural resources and being a key requirement for commercial exploitation [72].

The purified inhibitor was subjected to different temperatures and pHs in order to demonstrate its stability against these conditions. For this, a sample of GdTI (16 μg mL^{-1}) was incubated at temperatures between 40 and 100 °C or pHs between 2 and 12 (Section 2.6.3), after which the residual trypsin inhibitory activity was evaluated under standard assay conditions using BApNA as substrate and according to previously described in Section 2.4. In these trials it was possible to show that GdTI is stable in all the conditions tested, maintaining 100 ± 4% and 79 ± 5% of its average inhibitory activity at extremes temperatures and pHs, respectively (Fig. 5).

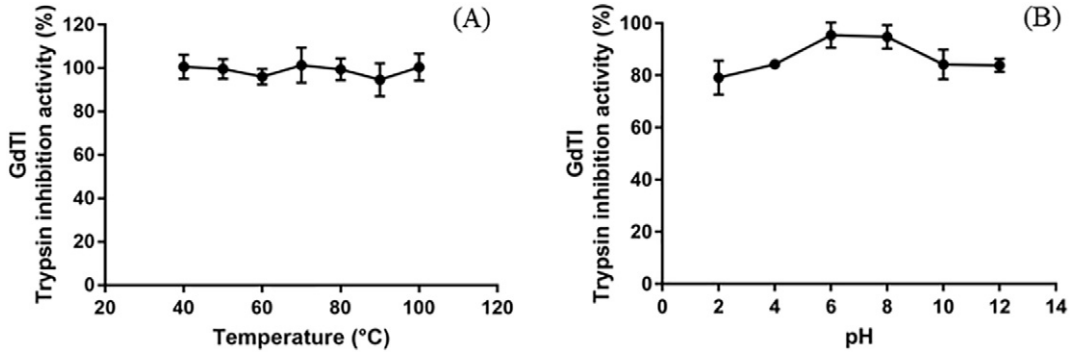

Fig. 5. Temperature and pH stability of *Geoffroea decorticans* trypsin inhibitor. (A) Residual trypsin inhibitory activity after incubation of GdTI for 60 min at temperatures from 40 to 100 °C. (B) Residual trypsin inhibitory activity after incubation of GdTI for 60 min at pHs from 2 to 12.

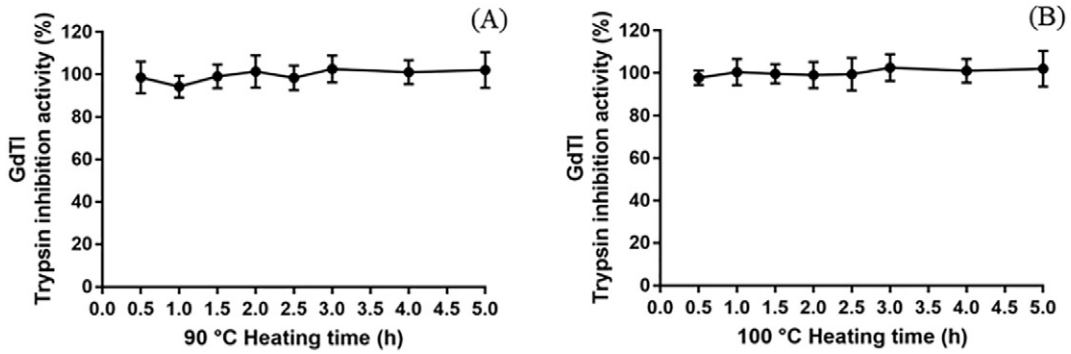

Fig. 6. Temperature stability at prolonged times for *Geoffroea decorticans* trypsin inhibitor. (A) Residual trypsin inhibitory activity after 90 °C incubation of GdTI for 0.5–5 h. (B) Residual trypsin inhibitory activity after 100 °C incubation of GdTI for 0.5–5 h.

Even more, the incubations at elevated temperatures (90 and 100 °C) for prolonged times were performed. Thus, it was possible to observe that even after incubating GdTI for 5 h at high temperatures, the trypsin inhibitory activity is not modified showing 99 ± 5% of its average activity (Fig. 6). Considering that the biochemical activity of a protein depends on the maintenance of its native conformation, is evident that GdTI is unusually stable protein resisting thermal treatments and pHs for long periods and preserving the native protein conformation with high bioactivity. Although this behaviour is comparable with several types of trypsin inhibitors, physicochemical stability for such prolonged times has not been previously evaluated (Table 4). It can be seen that *Luetzelburgia auriculata* Bowman-Birk Inhibitor maintains its trypsin inhibitory activity by 90% after 2 h of incubation at 98 °C [36], while Wild Soybean Trypsin inhibitor [51] and *Hyptis suaveolens* Trypsin Inhibitor [73] maintain their inhibitory activity after 60 min incubation at 90 and 94 °C respectively. These results are very promising since molecules of natural origin, with low molecular weight and physicochemical stability offer the advantage of being easily used in a wide range of applications, whether in the food industry, agricultural biotechnology, or pharmaceutical science [2].

After an extensive literature search, we could appreciate that only a few of the PPIs with high stability to extreme temperatures and pHs have biological activities. Most of these inhibitors have potential as biopesticides, with inhibitory activity on intestinal proteases of insects and/or inhibition of larval growth; HSTI [73], C11PI [74], CFPI [77] and RsBBI1 [79]. Other biological activities reported were antibacterial activity on *S. aureus* for LzaBBI [36] and anticoagulant activity on the extrinsic coagulation pathway for MpBBI [80]. Although PPIs of plant origin with high physicochemical stability would facilitate storage and application conditions, the biological activities of these PPIs have been poorly explored.

3.2.4. Biological activities

3.2.4.1. Anticoagulant activity. Although a wide variety of biological activities have been studied for trypsin inhibitors of plant origin (*e.g.*, anticancer, anti-inflammatory, anti-angiogenic, antimicrobial activity), activities as an anticoagulant and hypoglycemic agent were scarcely reported. The anticoagulant activity of the trypsin inhibitor of *Leucaena leucocephala* [81], *Enterolobium contortisiliquum* [82] and *Maclura pomifera* [80] has been reported for activated partial thromboplastin time, but not for prothrombin time. This indicates that the anticoagulant effect of these inhibitors is exerted on the intrinsic coagulation pathway. For GdTI both coagulation pathways were evaluated at increasing concentrations of inhibitor. We could observe that for the same concentration of GdTI there is a more pronounced decrease in coagulation time for aPTT compared to the time required for PT. For example, by adding 22 μM inhibitor in the PT test, we have observed a delay in the coagulation time from 18 s to 26 s, while in the aPTT test it is possible to delay the coagulation time, to the same concentration of GdTI, from 51 s to 110 s. Therefore, the trypsin inhibitor of chañar strongly delays the coagulation time for the intrinsic coagulation pathway, while for the extrinsic pathway higher concentrations are required to produce the same effect (Fig. 7). As a comparison, heparin—a drug commonly used in clinics—in a concentration of 333 μM produces a delay in coagulation time for the aPTT route from 18 to 300 s [83], while GdTI produces the same effect in concentrations of 26 μM. These results encourage future studies on the mechanism of action of GdTI and the development of *in vivo* assays that could be promising for a potential exploitation of this inhibitor as a naturally occurring antithrombotic agent. Our study not only highlights the anticoagulant capacity of GdTI but also demonstrates that it is the first plant inhibitor with an effect on the extrinsic coagulation pathway.

Table 4
Trypsin inhibitors from vegetable origin with temperature and pH stability.

Inhibitor name/s	*Ki* (M)	MW (kDa)	Temperature range (°C)	Incubation time (min)	IA after heat treatment (%)	pH range	Incubation time (min)	IA after pH treatment (%)	Reference
Cajanus cajan cv. ICP 7118 proteinase inhibitor (c11pi)	2.72×10^{-7}	8.38	20–80	30	85	2–12	60	85	[74]
Black gram protease inhibitor (BgPI)	3.07×10^{-7}	8.04	37–100	30	80	2–12	60	95	[75]
Phaseolus aureus inhibitor	N/D	16.6	30–90	30	99	1–13	30	75	[76]
Cratylia mollis trypsin inhibitor (CmTI)	1.4×10^{-9}	8.55	20–100	30	100	2–10	30	100	[56]
Clitoria fairchildiana proteinase inhibitor (CFPI)	3.3×10^{-10}	7.97	37–100	30	95	2–10	60	85	[77]
Maclura pomifera Bowman-Birk inhibitor (MpBBI)	6.6×10^{-8}	6.65	40–100	10	100	2–13	30	100	[78]
Rhynchosia sublobata Bowman Birk inhibitor (RsBBI1)	3.58×10^{-7}	9.97	40–100	30	95	2–12	60	100	[79]
Wild Soja trypsin inhibitor (WSTI)	4.6×10^{-9}	7.52	90	60	100	2–12	60	95	[51]
Hyptis suaveolens trypsin inhibitor (HSTI)	N/D	8.7	4–94	60	100	3–10.7	60	100	[73]
Luetzelburgia auriculata Bowman-Birk inhibitor (LzaBBI)	8.6×10^{-11}	17.3	98	120	90	2–11	180	80	[36]

Ki: dissociation constant, MW: molecular weight, IA: inhibitory activity, N/D: not determined.

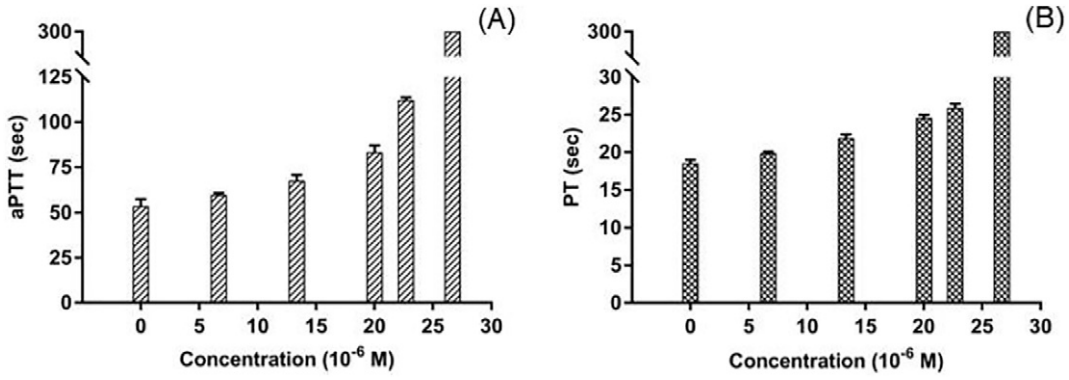

Fig. 7. Anticoagulant activity of GdTI on activated partial thromboplastin (aPTT) and prothrombin (PT). (A) Coagulation times with different concentrations of GdTI on aPTT. (B) Coagulation times with different concentrations of GdTI on PT.

3.2.4.2. Hypoglycemic activity. The inhibition of the α-glucosidase prevents the release of free glucose from complex carbohydrates, thereby lowering free blood glucose levels [84]. The serious side effects and the toxicity associated with some therapeutic drugs indicate the demand for diet-derived antidiabetic agents or approaches that are considered natural and safe. Hypoglycemic activity tests are not frequent in the characterization of biological activities for PPIs, being mostly informed for peptides product of protein hydrolysates [85,86]. In our work we demonstrated the inhibition of α-glucosidase by GdTI, showing an $IC_{50} = 0.18$ μM (equivalent to 1.21 μg mL^{-1}) (Fig. 8). Peptides from whey protein hydrolysates showed α-glucosidase inhibitory activity with $IC_{50} = 3.5$ mg mL^{-1} [85]. Similar levels of α-glucosidase inhibition were reported by Matsui & colleagues [87] and Yu & colleagues [88]. According to such results, it is possible to observe that GdTI strongly inhibits the enzyme α-glucosidase being able to have direct effects on the regulation of blood glucose levels. This effect added to the anticoagulant capacity above demonstrated for this inhibitor, makes it a molecule with great potential for the development of a natural antidiabetic drug.

4. Conclusions

It has been possible to isolate, purify and characterize a new trypsin inhibitor from chañar seeds, named GdTI. This inhibitor is, as far as we understand, not only the first inhibitor studied from chañar but it is also the first isolated and characterized protein in this plant species.

In this study, GdTI has proved to be a robust serine protease inhibitor whose biochemical and physicochemical characteristics different from those reported for this type of inhibitor make it a protein with potential biomedical and biotechnological applications. In this sense, GdTI stands out for presenting a very strong trypsin inhibitory activity ($Ki = 2.1$ nM), being among the most potent serine protease inhibitors reported so far. Likewise, GdTI has a very high physicochemical stability (80% residual trypsin inhibitory activity after 60 min at extreme pHs, 100% residual trypsin inhibitory activity after 5 h at 90/100 °C), being the first protease inhibitor reported that retains hyper stability during prolonged incubation times at extreme temperatures.

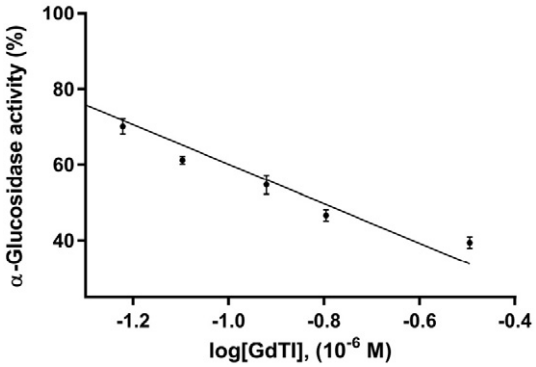

Fig. 8. Inhibitory activity of *Geoffroea decorticans* trypsin inhibitor against α-glucosidase.

Another interesting finding for its potential biomedical use is the evidence of the anticoagulant capacity of this inhibitor on both coagulation pathways, requiring lower doses of GdTI than heparin to produce the same delay in coagulation time for the aPTT pathway. In addition, surprisingly, GdTI represents the first report of a naturally occurring plant protease inhibitor on the extrinsic coagulation pathway. Finally, GdTI presented a strong inhibitory activity of the enzyme α-glucosidase ($IC_{50} = 0.18$ μM), a result that added to the anticoagulant activity is very promising for the potential application of this inhibitor as a natural antithrombotic and antidiabetic agent.

Thus, the results of our study show the discovery of a molecule of natural origin with unprecedented characteristics; GdTI is one of the most potent serine protease inhibitors found so far, with the highest thermostability reported in literature, with anticoagulant effect for both coagulation pathways and which also has hypoglycemic activity.

Actually, one of the most promising applications of serine protease inhibitors is in the field of diseases that are related to metabolic disorders, such as cardiovascular diseases, inflammation, and cancer, among others. The study of novel trypsin inhibitors will allow in the future the potential therapeutic approach over those diseases with trypsin overexpression, when all metabolic pathways where trypsin is implicated in the different tissues have been clarified. Due to the above, we propose to continue the study of GdTI focusing on its characterization at the molecular level and through the development of tests in *in vivo* models to evaluate in depth the biological activities presented here.

To sum up, the importance in the search of molecules that present these characteristics: high stability at pH and temperature, natural drug, low molecular weight protein, enormous power of action, biological activities of biomedical interest, all in the same molecule, makes GdTI in a very interesting and promising molecule for its biotechnological and commercial exploitation. In this sense, GdTI exerts its biological action at low doses, which would greatly decrease the chances of producing unwanted side effects. In addition, by requiring small doses to perform its function, the productive expenses for its isolation and purification are considerably reduced, which makes it more promising for industrial-level scaling. Finally, its high stability to pH and temperature makes GdTI a potential natural drug ideal for production in the pharmaceutical industry, not only due to low production costs, but also does not present special storage needs, being available for use in geographic regions of extreme, unfavourable and adverse climates.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijbiomac.2020.01.214.

CRediT authorship contribution statement

Juliana Cotabarren: Investigation, Formal analysis, Writing - original draft, Methodology, Writing - review & editing.**Daiana Judith Broitman:**Investigation, Formal analysis, Writing - original draft. **Evelina Quiroga:**Investigation, Formal analysis, Writing - original draft.**Walter David Obregón:**Investigation, Formal analysis, Writing - original draft, Methodology, Writing - review & editing.

Acknowledgments

J. Cotabarren is a postdoctoral fellow from the Argentine Council of Scientific and Technical Research (CONICET); E. Quiroga and W.D. Obregón are members of the Researcher Career Program of the CONICET.

The authors acknowledge the support from Universidad Nacional de La Plata (Argentina) under grants PPID X/014 and PPID X/038, Universidad Nacional de San Luis (Argentina) under Project 22116, CONICET (Argentina) under Project PUE 229201601 00026CO, and the National Agency of Scientific and Technological Promotion (Argentina) under Project PICT-2016-4365.

Declaration of competing interest

The authors declare no competing financial interest.

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