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Biochemical characterization and phytotoxic activity of protein extract from *Euphorbia tirucalli* L

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ABSTRACT

Ethnopharmacological relevance: Euphorbia tirucalli L., a tropical and subtropical plant, also known by the popular name avelós, has been used in folk medicine against many diseases as rheumatism, asthma, toothache, and cancer. Studies have shown that natural compounds contained in this plant species may be associated with these functions. However, little is known about its potential toxicity.

Aim of the study: Several proteins conduct biological functions, in particular, proteinases, play a crucial role in many mechanisms of living beings, including plants, animals and microorganisms. However, when poorly regulated, they can generate consequences, such as the non-production of certain substances, or even the abnormal multiplication of cells, which leads to tumors. On the other hand, by regulating these enzymes, proteinase inhibitors act by reducing the activity of proteinases, thus preventing their malfunction. The objective of this work was to evaluate the toxicity of the protein extract of *E. tirucalli* and to purify a protease inhibitor that may be associated with the biological medicinal functions of the plant.

Materials and methods: The cytotoxic and mutagenic properties of the protein extract produced from the stem of avelós was investigated using the Ames test. The protein extract was also submitted to a protease inhibitor purification process using the gel filtration chromatography technique and the purified protein was biochemically characterized.

Results: A protease inhibitor, called tirustatin, was isolated 1.84-fold by Biogel P100. The calculated molecular mass of the isolated protein is 25.97 kDa. The inhibitor was stable at pH 3–10, with pronounced activity at pH 6. Thermostability was observed even at elevated temperature (100 °C) with inhibitory activity increased by 1.14-fold compared to inhibitor activity at room temperature. Incubation at basic pH values for up to 60 min caused little reduction (0.25-fold) in the papain inhibitory activity of tirustatin. The stoichiometry of the papain-tirustatin interaction was 1.5: 1 and 28.8 pM of the inhibitor effected 50% inhibition. With an equilibrium dissociation constant of 8.74 x 10-8M for the papain enzyme, it is possible to evaluate the isolated protein as a non-competitive inhibitor. In addition, the protein extract of *E. tirucalli* even at the maximum concentration used (20 μg/mL), did not show a cytotoxic and mutagenic profile in a bacterial model.

Conclusion: The results presented in this work provide data that reinforce the idea of the potential use of proteins produced in *E. tirucalli* as pharmacological and biotechnological agents that can be exploited for the development of efficient drugs.

1. Introduction

The species *Euphorbia tirucalli* L., known as “avelós”, is a plant of the Euphorbiaceae family. The species originates from the eastern region of the African continent and is endemic in some countries such as Uganda, Ethiopia, and Angola (Mwine et al., 2013; Van Damme, 1989).

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Succulent, with tiny leaves and flowers (rarely seen) the plant is known to be laticiferous, a common feature of this family (Mali and Panchal, 2017). It grows in dry areas and is generally used as a living fence for demarcating boundaries (Mali and Panchal, 2017).

Despite being known as a toxic plant (Shlamovitz et al., 2009), *E. tirucalli* plants has been widely used in folk medicine (Gupta et al., 2013). In countries as India, Indonesia and Malaysia, and Brazil, the latex produced by the plant is used for the most diverse diseases, including cough relief, wart removal, and in treatment of sexual impotence, epilepsy and cancer (Mwine et al., 2013). In the last years, studies involving this plant species confirmed its antibacterial and larvicidal (Parekh et al., 2005; Rahuman et al., 2008), antioxidant and hepatoprotective (Jyothi et al., 2006) and antitumor (Munro et al., 2015). For this reason, the plant is target of biochemical investigations that have already resulted in the isolation of several substances (Martins et al., 2020; Palharini et al., 2017). On the other hand, little is known about the biotechnological potential of proteins produced by *E. tirucalli* plants.

Studies show that the latex produced in avelós plants is toxic, as it can cause irritation to mucous membranes, eyes and skin (Mwine et al., 2013). However, there are no reports in the literature about the cytotoxic or mutagenic potential of the protein extract of *E. tirucalli*, which makes this study even more necessary.

In this sense, seeking more information about this species, in the present work, aiming to analyze the toxicological and mutagenic potential of the proteins contained in *E. tirucalli* plants, we detected the activity of a protease inhibitor (PI) constitutively produced by the plant. PIs are known to keep the balance between the physiological processes of proteolysis and the synthesis of proteins in plants. In addition, these inhibitors are important molecules related to plant defense mechanisms against attack by herbivorous insects and pathogenic microorganisms. (Shamsi et al., 2016). PIs are commonly grouped into several families based on the similarity sequences and structural relationships. Such families include Kunitz, Bowman–Birk, potato I and II, cysteine protease inhibitors, serpins, α-amylase/trypsin inhibitors, squash trypsin inhibitors, mustard trypsin inhibitors, aspartic protease inhibitors, and metal-carboxypeptidase inhibitors (Consiglio et al., 2011).

Our data has demonstrated the safe concentrations concerning pharmacological use and the process of isolation and partial biochemical characterization of a phytocystatin produced for *E. tirucalli* plants.

2. Material and methods

2.1. Plant materials

Leaves and stems of *E. tirucalli* was harvested from the city of São João da Barra, RJ, Brazil (12m altitude, 21°38′56″ S and 41°3′9″ W) in 2019. The plant was authenticated by one of the authors (C.L.S.J), and a voucher specimen deposited at the Herbarium Prof. Jorge Pedro Pereira Carauta – HUNI of Universidade Federal do Rio de Janeiro (no. HUNI 5769). The plant identification was based on macro-morphological analysis of both the vegetative and reproductive structures of *E. tirucalli* under a stereoscopic microscope. The leaves and stems were cut and stored at −20 °C until required.

2.2. Extraction and recovery of proteins

Protein extraction was performed according to the methodology described by Tavares et al. (2021). Briefly, 20 g of sample was added to 80 mL of ice-cold extraction buffer (50 mM sodium phosphate, pH 6.0), homogenized in 10% (w/w) of insoluble polyvinylpyrrolidone (PVP). The prepared homogenate was incubated at 4 °C on a rotary shaker for 90 min. The homogenate was then filtered through a qualitative paper filter (80g, 22 μm), and then it was centrifuged at 15,000×*g* for 30 min at 4 °C. The recovered crude supernatant (with 30% yield) was used for the following experiments.

2.3. Protein determination

Protein concentration was determined according to the method of Bradford (1976) using the Bradford assay kit and bovine serum albumin (BSA) as standard protein according to the manufacturer's instructions (BioRad).

2.4. Protease inhibitor activity assay

Activity of protease inhibitor was detected according to Siqueira-Júnior et al. (2008). *E. tirucalli* crude extract was added to 2 μg of papain enzyme and the volume was made to 315 μL by adding 250 mM sodium phosphate buffer, 2.5 mM EDTA, 25 mM β-mercaptoethanol (pH 6.0). After incubation at 37 °C for 10 min, the reaction was started by the addition of 35 μL of 5 mM BANA (SIGMA). The reaction was then stopped by adding 500 μL of 2% HCl/ethanol, after 30 min of incubation. Then, the color was obtained by adding 500 μL of 0.06% p-dimethylaminocinnamaldehyde prepared in ethanol, keeping the mixture for 30 min. The absorbance of released p-naphthylamine was taken at 540 nm. As an experimental control, only papain in 350 μL of phosphate buffer was used. Blanks were prepared under the same conditions described above, without the addition of papain, which was only added after the stop solution. Inhibitor activity was calculated by the amount of plant protein required to decrease absorbance by 0.01 at 540 nm, which is considered as one unit of papain inhibition (UI) and expressed as papain inhibitor units per mg plant protein.

2.5. SDS-polyacrylamide gel electrophoresis and immuno-blot analysis

E. tirucalli samples were analyzed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) using 10% (w/v) acrylamide, according to Laemmli (1970). The transfer of proteins to nitrocellulose membranes was carried out as described by Siqueira-Júnior et al. (2008). Membranes were probed with immune serum against tomato cystatin were developed using Protein A-peroxidase (Sigma) and True Blue Peroxidase Substrate kit (KPL) following the manufacturer's instructions.

2.6. Purification of the tirustatin

The prepared crude extract from *E. tirucalli* was brought to 30% saturation (w/v) with a gradual addition of solid ammonium sulphate [$(NH_4)_2SO_4$] saturation at 4 °C with vigorous stirring for 90 min. The resulting precipitate (F30) was recovered by centrifugation at 15,000×*g* for 30min at 4 °C. The pellet was solubilized and dialyzed overnight at 4 °C against ultrapure water. The extract was centrifuged at 10,000×*g* for 10 min at 4 °C and the supernatant was applied to a size exclusion chromatography on P100 biogel (BioRad) column pre-equilibrated with 20 mM Tris-HCl (pH 6.0). The column was fixed with flow rate of 15 mL/h. Proteins were detected by monitoring absorbance at 280 nm, and fractions were assayed against and papain. The purified cystatin obtained was named as Tirustatin.

2.7. Thermal and pH stability of the tirustatin

To determine the thermal stability, 5 μg of the purified inhibitor was incubated with 250 mM sodium phosphate buffer, 2.5 mM EDTA, 25 mM β-mercaptoethanol (pH 6.0) at various temperatures ranging from 20 to 100 °C in a water bath for 120 min. After cooling rapidly in ice cool water bath samples are checked for residual activity against papain as described previously. As a variation of the assay, the effect of heat treatment on inhibitory activity of tirustatin was also investigated after boiling the inhibitor for different incubation times (10, 30, 60 and 120 min) under the same conditions described above.

To assess the stability of the inhibitory activity at different pHs, 5 μg of boiled inhibitor (for 120min) was dissolved in an appropriate buffer

(250 mM): citrate buffers (pH 3–5), phosphate buffer (pH 6–8) and Tris-Buffers of HCl (pH 9–11). The inhibitory activity of the protein against papain was again analyzed as described above, followed by 24 h of incubation at each pH at room temperature.

2.8. IC_{50} analysis of inhibition and determination of the inhibition constant (K_i)

The concentration of tirustatin capable of reducing the papain activity by 50% (IC50) was determined by incubating constant concentrations of papain (2 μg) and BANA (5 mM) substrate with different concentrations of this inhibitor (0.1 at 5.0 μg). The increasing percentages of inhibition observed, and the concentrations used were plotted on a graph and the IC_{50} was calculated using simple linear regression. The determination of *Ki* values and the evaluation of the inhibitory activity of tirustatin against papain using BANA as substrate were based on the methodology described by Jacinto et al. (1998).

2.9. Salmonella mutagenic assay

The mutagenicity of *E. tirucalli* proteins was assessed using the Salmonella/microsome assay following the protocol adapted by Tavares et al. (2021) with slight modifications. *Salmonella typhimurium* strains (TA97, TA98, TA100, TA102 and TA104) were incubated (16h, 37 °C, 60 rpm) in 10 mL of LB (Luria-Bertani) liquid medium (tryptone 1%; yeast extract 0.5%; NaCl 1%) with ampicillin (8 mg/mL) and tetracycline (8 mg/mL), only for TA102, to obtain the stationary growth cultures of *S. typhimurium* for used in the test. Briefly, 100 μL of each stationary culture strain (1–2 x 10^9 cells/mL) were preincubated with 100 μL of crude extract at different concentrations (20 μg/μL; 2 μg/μL 0.2 μg/μL; 0.02 μg/μL; 0.002 μg/μL) diluted in 0.9% NaCl, or positive and negative controls, and 500 μL of 0.2 M sodium phosphate buffer (pH 7.4) at 60 rpm, at 37 °C. The amount of inhibitor from crude extract estimated in each tester concentrations for mutagenicity and cytotoxicity is 136 μg; 13.6 μg; 1.36 μg 0.136 μg and 0.014 μg. After 20 min, 2 mL top agar (0.7% agar, 0.5% NaCl, 10% histidine/biotin) at 40 °C were added to the test tubes and the final mixtures were poured into Petri dishes containing Vogel-Bonner agar medium (1.5% agar, 2% Vogel-Bonner salt solution, 2% glucose). These final mixtures were incubated at 37 °C for 72 h. Each tester strain was assayed in triplicate, and the number of His^+ revertant colonies was counted for each tester strain and treatment group. The positive controls used in the Ames assay were 4- nitroquinoline 1-oxide (CAS: 56–57-5) at 0.01 μg/mL for TA97, TA98 and T100; and methyl methanesulfonate (MMS) at 0.25 μg/μL for TA102 and 2.5 μg/μL for TA104. According to OECD, Guidelines 471 (OECD, 1997), the mutagenic response of crude extract was considered positive when the number of revertant colonies in the test was at least twice the number of spontaneous revertants. Tests were carried out in triplicates, being repeated three times, and the results analyzed statistically by ANOVA and Student's *t*-test, to verify significant statistical differences between negative and tested concentrations under the same experimental conditions (Stankevicins et al., 2008).

2.10. Survival experiments

To determine the cytotoxic effects of crude extract, 100 μL of solution containing bacteria in stationary phase (1–2 x 10^9 cells/mL) were incubated at 37 °C, in contact with the samples, or positive and negative controls, under the same conditions described for the test of Ames. After 20 min preincubation, the assay mixtures were diluted in 0.9% NaCl (w/v) to a final concentration of 1–2 x 10^2 cells/plate and plated on LB agar medium (1% tryptone; 0.5% yeast extract; 1% NaCl; 1.5% agar). The plates were incubated for 24 h at 37 °C, and the colonies were counted, and the percentage of survival was calculated in relation to the negative control. Samples were considered a toxic concentration when survival rates were less than 70% compared to the control group.

2.11. Statistical analysis

All tests were conducted in triplicates and repeated three times. The results were calculated as mean along with standard error values, and the variance analysis was established by ANOVA. Significant differences between negative and tested concentrations under the same experimental conditions were calculated using Student's *t*-test, with significance level reported at $P \leq 0.05$ (Stankevicins et al., 2008).

3. Results

3.1. Detection of protease inhibitors in plant extract

In this study, following the extraction process, both the crude protein extract and the F30 fraction were submitted to the evaluation of inhibitory activity against papain, according to the methodology described by Siqueira-Júnior et al. (2008). Samples showed high inhibitory activity against papain enzyme with a mean of 737,44 UI/mg of protein (Fig. 1A).

SDS-PAGE of the crude extract showed a profile with a higher concentration of proteins with molecular weights between 21 kDa and 45 kDa (Fig. 1B). Western blotting analysis, immunostaining the proteins with an anti-tomato cystatin antiserum, showed a positive signal with two proteins of 38 and 26 kDa respectively, indicating the presence of cysteine proteinase inhibitors in avelós plants (Fig. 1B).

3.2. Purification of cystatin

Cysteine protease inhibitors was partially purified from avelós stems according to the method described by Botelho-Junior et al. (2008) with some modifications. Crude extract was subjected to ammonium sulphate precipitation (0–30% and 30–60%) to remove unwanted proteins. To isolate the phytocystatins, F30 fraction (0–30% $[(NH_4)_2SO_4]$ saturation), that showed the greatest inhibitory activity compared to other fractions, was submitted to size exclusion chromatography on P100 biogel (Fig. 2). The fractions 10 to 14 showed a percentage of papain inhibition between 30 and 45%. For this reason, these fractions were pooled, concentrated by ultrafiltration, and used for further analysis (Fig. 2).

The methodological procedure resulted in purification (1.84-fold) with a 137.84% yield (Table 1).

3.3. Molecular weight of cysteine protease inhibitors

Analysis of the fraction eluted by SDS-PAGE indicates the presence of only one protein band showing the purification of the protease inhibitor. A polypeptide band with a molecular mass of 25,970 Da in reductive SDS-PAGE (Fig. 2-inset) that cross-react with the antibodies produced against tomato cystatin have been named as *Tirustatin*.

3.4. Thermal and pH stability

The purified inhibitor is thermal stable at elevated temperatures (100 °C). When the inhibitor was incubated at temperatures above 40 °C, in addition to maintaining its antipapain activity, it also slightly increases its percentage of inhibition (Fig. 3A). Even at elevated temperatures (boiling at 100 °C) the inhibitory activity of tirustatin is maintained after 2 h of heat treatment (Fig. 3B). A cystatin isolated from papaya seeds by our group (Jeunon, 2015), used in these tests as a control, lost its inhibitory activity after heat treatment unlike the inhibitor purified from avelós stems (Fig. 3- inset).

Pre-incubation in the pH range 3–11 for 24 h did not affect the inhibitor (Fig. 4). Tirustatin stability was verified over a wide pH range, recording a maximum inhibitory activity at pH 6. At more alkaline pH (10) there was a slight decline of 24% in inhibitor activity against the papain enzyme.

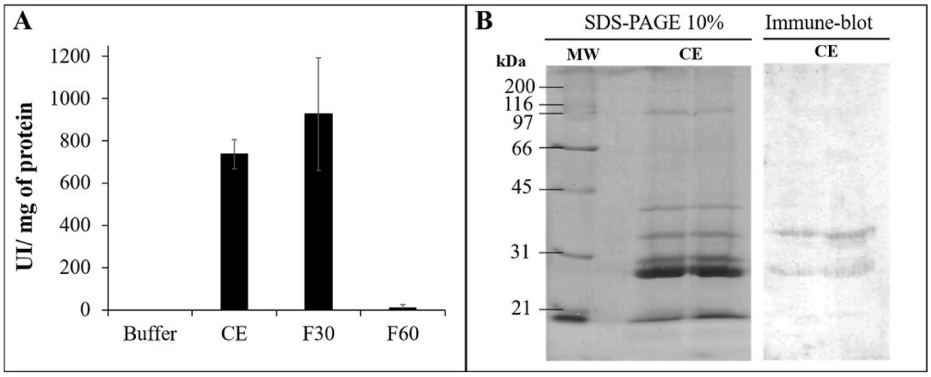

Fig. 1. Proteinase inhibitor detection in *E. tirucalli* plants. (A) Inhibitory activity (specific activity = UI*/mg of protein) in crude extract (CE) and protein fractions of *E. tirucalli* fresh stems. F30 and F60 represent the fractions obtained in respective saturation ranges of 0–30% and 30–60% by precipitation with (NH4) 2SO4. From each sample, 50 mg of crude stem extract were incubated with 5 mg of papain and the proteolytic activity evaluated using the substrate BANA. Data are an average of three independent experiments and the mean ± S.E was reported. *One unit of inhibitory activity (UI) was defined as the amount of inhibitor that decreased absorbance by 0.01, under assay conditions. (B) SDS–PAGE and Western blot analysis. Protein samples were analyzed on SDS-PAGE 10%. Then they were blotted and immunoreacted with anti-tomato cystatin antiserum. Lane CE, crude extract obtained from *E. tirucalli* stems. lane MW- molecular weight marker. Proteins were probed with 10,000-fold diluted immune serum against cowpea cystatin.

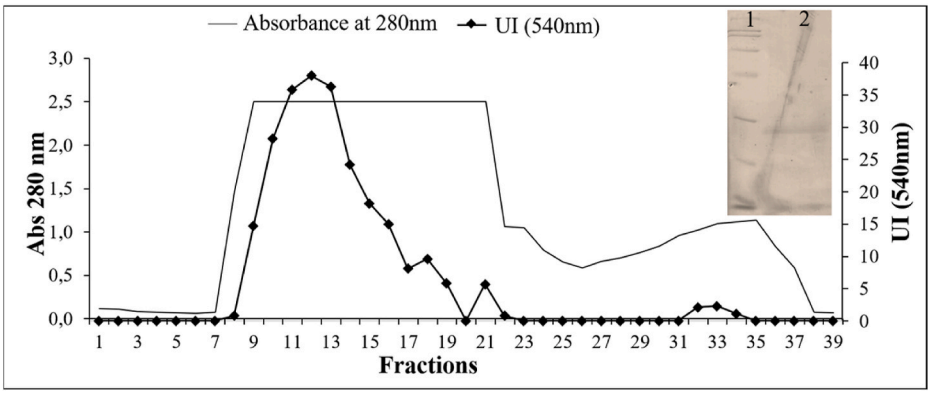

Fig. 2. Chromatographic elution profile of F30 fraction by gel filtration on biogel P100 resin (Bio-Rad). At a flow rate of 15 mL/h, the column was pre-equilibrated with 20 mM Tris-HCl buffer pH 6.0. Fractions of 2 mL were collected and evaluated for protein concentration at 280 nm and for inhibitory activity against papain. UI represents a unit of inhibitory activity, as described in material and methods. Fractions 10 to 14 were pooled for further analysis. Inset SDS – PAGE showing the fractions obtained during the purification process: lane 1 - molecular weight marker, lane 2 - purified tirustatin.

Table 1
Purification steps of protease inhibitors from *E. tirucalli* stems.

Sample	Total protein (mg)	Protease Inhibitor Activity (UI[a])	Specific inhibitor activity (UI/mg)	Yield of activity (%)	Fold of purification
Crude Extract	0.32	25.5	80.02	100	1
$(NH_4)_2SO_4$ fraction (0–30%)	0.4	41.93	105.01	64.43	1.31
Gel filtration by P100 – Biogel	0.41	60.65	147.93	137.84	1.84

[a] One UI (unit of inhibitory activity) was defined as the amount of inhibitor that decreased absorbance by 0.01, under assay conditions.

The data obtained for the studies conducted on protease – tirustatin interaction is depicted in Fig. 5. Different concentrations (11–550 *p*mol mL^{-1}) of purified PI were used to determine the IC_{50} of papain. The assayed results showed linear inhibition of papain activity with increase of inhibitor until saturation was achieved. Tirustatin inhibited papain with an IC_{50} of 28.8 *p*M (Fig. 5) suggesting that this inhibitor has significant inhibitory potential against papain.

3.5. Kinetic analysis of inhibitor

Fig. 6 shows the Lineweaver–Burk double reciprocal plots of different concentrations of BANA versus the papain activity in the presence and absence of two concentrations of purified tirustatin. This plot shows that with the increase of inhibition, both V_{max} and K_m decrease indicating a non-competitive inhibition mechanism for tirustatin. From the titration the inhibitory constant (K_i value) for tirustatin for papain was calculated to be 8.74 x 10^{-8} M under the assay conditions. This low K_i value indicates that tirustatin is a potent inhibitor of cysteine protease.

3.6. Toxicological studies

Table 2 demonstrates the results of the evaluation of the cytotoxic and mutagenic potential of *E. tirucalli*. As results, the extracted proteins did not elicit mutagenic (MI < 2) or cytotoxic (survival rates <70%) responses to none of the *Salmonella typhimurium* strains used in experiments.

4. Discussion

Among the proteins contained in *E. tirucalli* crude extract, the activity of PIs was detected. These proteins are well known to have an important role in the plant defense mechanism (Volpicella et al., 2011). In addition to its roles in plant defense, PIs have been examined extensively as therapeutic agents. Interestingly, several plant PIs are under further evaluation in *in vitro* clinical trials to test antitumoral and antiviral activities (Li et al., 2017; Srikanth and Chen, 2016). The partial inhibition of the proteolytic activity of papain when incubated with CE indicates the presence of protease inhibitors in *E. tirucalli* stems. Among proteins extracted two proteins reacted with antibodies prepared against tomato cystatin, indicating that papain inhibitory activity may be associated with the presence of phytocystatins in the extract. A cysteine protease

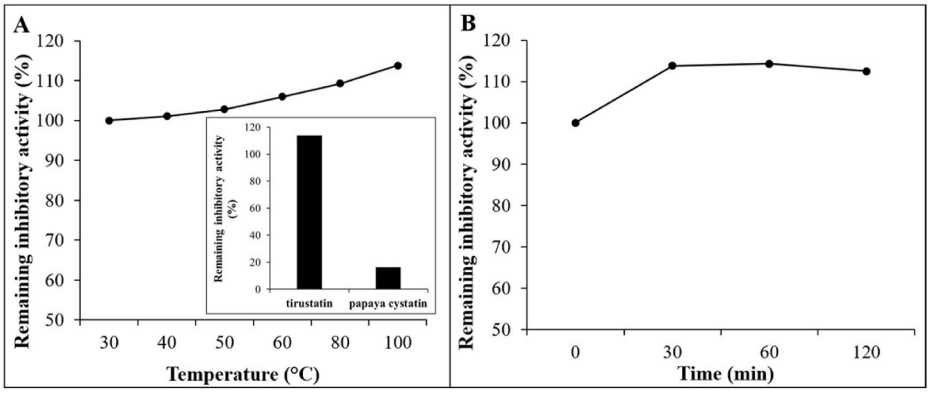

Fig. 3. Thermal stability of purified tirustatin. (A) The isolated inhibitor was incubated for 30 min at different temperatures (30–100 °C). (B) Purified inhibitor was also stored at 100 °C for varying time intervals. After rapidly cooling the protein, inhibitory activity of tirustatin against papain was analyzed. Each point shows the average of two experiments. Detail: effect of boiling for 30 min on tirustatin activity compared to a papaya seed cystatin.

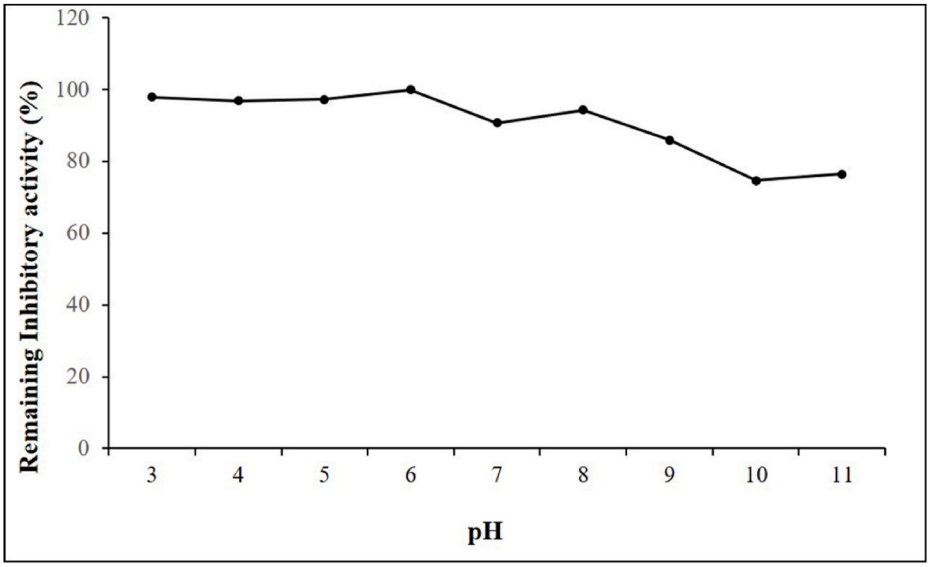

Fig. 4. pH stability of purified protein after incubation at the indicated pH. Tirustatin was pre-incubated with various buffers at 50 mM concentration: citrate phosphate buffer (pH 3.0–5.0); sodium phosphate buffer (pH 6.0–8.0) and Tris buffer – HCl (pH 9.0–11.0) for 24 h at 37 °C. After the incubation period, the inhibitory activity against papain was measure.

inhibitor with molecular mass of 25.97 kDa which was named *Tirustatin* was purified from avelós plants by ammonium sulphate and size exclusion chromatography on P100 biogel. leading to a final yield of 137.84% with a fold purification of 1.84 of tirustatin. Purity of the isolated tirustatin was ascertained by the emergence of a single peak in the elution profile with significant papain inhibitory activity and was verified from SDS-PAGE. Plants of genus *Euphorbia* have been investigated for the presence of proteases and lectins (Domsalla et al., 2010; Souza et al., 2011), but to our knowledge, this is the first report of a cystatin isolated from *E. tirucalli*. Some phytocystatins with molecular mass of 26 kDa, 13.9 kDa, 11.2 kDa and 9.3 kDa have been reported from *Brassica alba* seeds (Ahmed et al., 2016), *Hordeum vulgaris* seeds and *Helianthus annuus* seeds, respectively.

Displaying wide pH amplitude, tirustatin proved stable under conditions ranging from highly acidic to highly alkaline with a slight decrease in activity at pH > 9. Surprisingly, the isolated inhibitor shows high stability to heat treatment, with its activity increased at extreme temperatures. Maximum activity is achieved after boiling for 2 h. A phytocystatin isolated from barley seeds has been described for its stability at elevated temperatures (Abd El-latif, 2015), but until now no increase in activity has been reported after treatment for any phytocystatin, which reinforces the potential biotechnological use tirustatin.

Plant protease inhibitors have been described as having a reversible as well as irreversible mechanism. The kinetic study of tirustatin purified from *E. tirucalli* shows that it has a reversible mechanism of action. It was also observed that the stoichiometry of tirustatin and papain interaction takes place in a 1:1.5 M ratio and the amount of this PI needed for the 50% papain inhibition was 28.8 *p*M. The kinetic analysis revealed a non-competitive type of inhibition with a K_i of 8.74 x 10^{-8} M. The K_i value obtained for papain are quite comparable to the ones reported for corn cystatin I (3,7 x 10^{-8} M) (Abe et al., 1994), oryzacystatins I (3,2 x 10^{-8} M) (Kondo et al., 1990) and two soybean cystatins induced by methyl jasmonate and wounding (5.7 x 10^{-8} M and 2.1 x 10^{-8} M) (Zhao et al., 1996). Tirustatin is found to be effective inhibitor of papain as indicated by their low K_i value. Other phytocystatins such as tomato (Wu and Haard, 2000), almond (Siddiqui et al., 2016) were also reported to be non-competitive inhibitors against papain.

The use of medicinal plants has been occurring for centuries and increases in parallel with the number of reports of side effects and other problems, mainly due to the indiscriminate use and the insufficient number of studies that prove their biological effects, dosage and toxicity

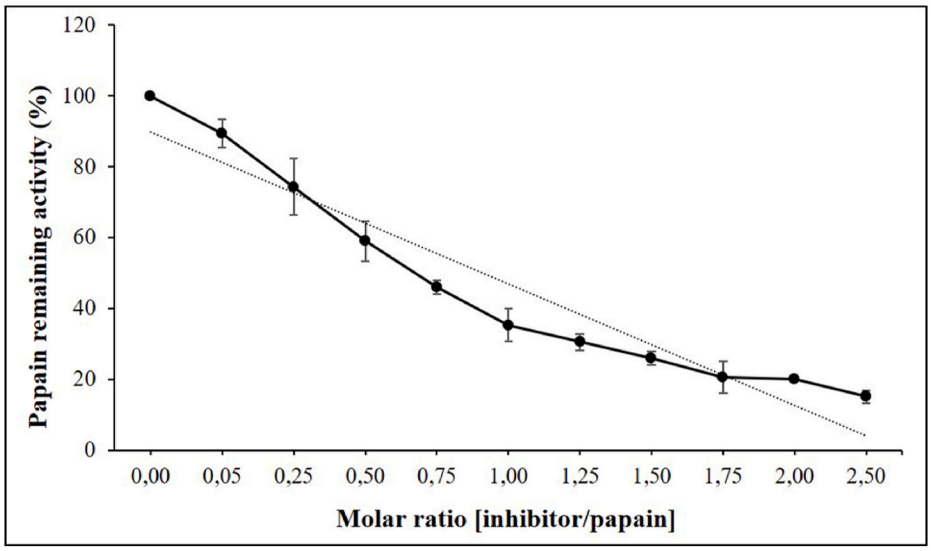

Fig. 5. Titration curve of papain inhibition by tirustatin. The inhibitor at increasing concentrations was added to a fixed concentration of papain (85.47 *p*moles). The residual activity of papain was determined as described in section material and methods. Each point is the average of three assays.

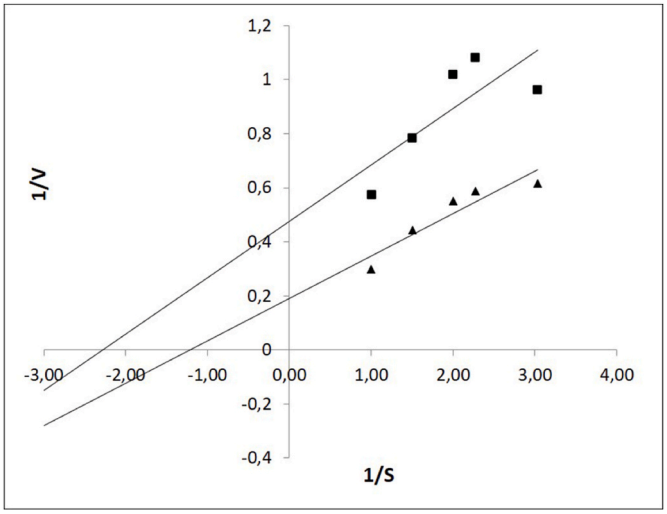

Fig. 6. Enzyme inhibition kinetics of tirustatin. Lineweaver–Burk plot (or double reciprocal lot) representing the inhibition of papain activity at different concentrations of the BANA substrate in the presence of two fixed concentrations of the phytocystatin. 23 pmoles tirustatin (filled triangle), 77 pmoles tirustatin (filled square). The rates of each reaction were calculated, and the reciprocal values of these velocities are plotted against reciprocal concentrations of papain as a Lineweaver–Burk plot.

(Ekor, 2014). The main record of avelós aqueous extract use is related to oral administration, although other methods of extraction have several uses, including topical (Costa, 2011; Varricchio et al., 2008). For this reason and due to widespread use of the aqueous extract of this vegetable in traditional medicine, in several countries, the Ames Test was carried out to assess the cytotoxicity and mutagenicity of avelós crude extract.

Interestingly, the Ames test can investigate the ways of DNA damage by strains analysis. The TA97 and TA98 are frameshift detect mutation strains, otherwise TA100, TA102 and TA104, can detect base pair substitution. On the first case, a single or double strand break should be included in the damage, in order to request a recombination repair and the base insertion. On the second case, for the base substitution we need a depurination, deamination of cytosine and 5-methyl cytosine, and exposure to endogenous alkylating agents and reactive oxygen species that the base substitution should be important.

The resulting nicks and gaps in DNA can lead to the formation of double strand breaks (DSBs), which are estimated to occur spontaneously in mammalian cells at a rate of 50 DSBs/cell/cell cycle or 1 DSB/10^8 base pairs/cell cycle (Vilenchik and Knudson, 2003). In bacteria, repair of DNA damage, including DSBs, which results in stalled or collapsed replication forks, is mediated by the RecA protein in conjunction with either the RecBCD or RecFOR pathways (Cox, 1999).

Our study indicated that proteins present in the avelós crude extract can promote an antimutagenic effect that effectively inhibit spontaneous mutations frequency in *Salmonella typhimurium* TA98 by 10–40% and that compound, significantly, reduced mutations at GC sites but not at AT sites. Proteins from avelós crude extract is able to protect the cells directly or indirectly through modulating intracellular protein, which elicits recombinational repair. This activation of recombinational repair can permits repair of other DNA lesions, thus reducing the frequency of spontaneous mutation.

Since crude protein extract is complex, it is necessary to separate molecules to assess the cytotoxic potential of each one. For this, further studies with purified proteins of the extract should be conducted. In addition, no mutagenicity was detected in the strains evaluated by the crude extract. Thus, an extract with cytotoxic activity but not mutagenic potential may indicate the possibility of the presence of substances with anticarcinogenic action in the plant, which corroborates the traditional registered use of this species (Gupta et al., 2013). The present study sheds some light on pharmacological potential of *E. tirucalli* plants and on the use of cystatin present in stem of these plants in production of new drugs.

5. Conclusions

Our results demonstrated that proteins from the crude aqueous extract of *E. tirucalli* stems does not induce mutagenic or cytotoxic responses, testing with 5 strains of *Salmonella* recommended for testing, in the absence of exogenous metabolism, and can be an antimutagenic response by decrease in the number of spontaneous mutagenesis, in TA98 strain. Such results indicate not only the biotechnological potential to be studied in the plant, through the detection of new bioactive molecules, but also enable the continuation of toxicological investigations of the plant extract, to improve safety in its use. In addition, our study demonstrates the potential of this plant as a source of cysteine protease inhibitor that can be used as therapeutic agents against

Table 2
Induction of His + revertants in *S. typhimurium* strains by E. tirucalli protein extract in reverse mutation assay (Ames test), without (−S9) metabolic activation.

Strain	*E. tirucalli* (μg/plate)	M.I.[a]	*His*$^+$ ± SD[b]	% Survival[c]
TA97	0	1.0	80 ± 23.3	100
	0.002	0.8	66.5 ± 2.1	100
	0.02	1.2	98 ± 8.5	95.6
	0.2	1.3	101 ± 11	87.2
	2	1.2	95 ± 22.2	100
	20	1.0	76 ± 2.8	100
	CP	5.4	432 ± 10	
TA98	0	1.0	24 ± 2.4	100
	0.002	0.9	22.7 ± 3.8	100
	0.02	0.8	18.5 ± 3.5	100
	0.2	0.6	14.3 ± 2.9	92.2
	2	0.7	16.3 ± 0.6	94.1
	20	0.7	16.5 ± 10.6	100
	CP	17.4	244.0 ± 10	
TA100	0	1.0	72 ± 25.5	100
	0.002	1.2	85 ± 14.1	83.6
	0.02	1.1	76.5 ± 16.3	74.6
	0.2	1.1	82 ± 21.2	77.6
	2	1.3	94.5 ± 47.4	71.6
	20	1.4	97.5 ± 6.4	65.7
	CP	11.3	815 ± 0	
TA102	0	1.0	348.5 ± 23.3	100
	0.002	1.0	343 ± 1.4	100
	0.02	1.0	350.7 ± 75	92.4
	0.2	0.8	281.7 ± 40.2	91.1
	2	0.8	289.5 ± 47.4	100
	20	0.8	295 ± 8.5	100
	CP	2.7	930 ± 0	
TA104	0	1.0	380 ± 9.9	100
	0.002	0.9	327 ± 3.1	100
	0.02	0.9	333 ± 11	100
	0.2	1.0	370 ± 3.6	100
	2	1.1	413 ± 15.4	100
	20	0.9	355 ± 20.5	100
	CP	4.1	1560 ± 16	

[a] Mutagenic index: No. of *His* $^+$ induced in the sample/number of spontaneous *His*$^+$ in the negative control (DMSO).
[b] *His*$^+$/plate: mean values of at least 3 replicate plates.
[c] Percent survival relative to the negative control: toxicity is considered when percent survival <70% related to the control group. Positive controls are described in the Experimental section. Statistical analysis (ANOVA and Student *t*-test) showed that differences were not significant ($P > 0.05$).

proteases, in food preservation, against proteolytic bacteria, as well as in agriculture as a natural insecticide. We conclude that *E. tirucalli* protease inhibitor is a valuable source of a potential cysteine protease inhibitor with immense application in biotechnology and in biomedical field.

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CRediT authorship contribution statement

Ana Carolina Pereira Milhm: Methodology, Formal analysis, Investigation, Validation. **Luiz Felippe Sarmento Bonet:** Methodology, Formal analysis, Investigation, Validation. **Claudia Alessandra Fortes Aiub:** Project administration, Conceptualization, Resources, Methodology, Supervision, Writing – review & editing. **César Luis Siqueira Junior:** Project administration, Conceptualization, Resources, Methodology, Supervision, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare no conflicts of interest.

Abbreviations

ANOVA	analysis of variance
BANA	N-benzoyl-arginine-naphthylamide
BSA	bovine serum albumin
CE	Crude Extract
EDTA	Ethylenediamine tetraacetic acid
F30	30% saturation fraction
MI	mutagenic index
MMS	methanesulfonate
OD	optical density
OECD	Organization for Economic Co-operation
PVP	polyvinylpyrolidone
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
UI	unit of inhibitory activity

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