JcTI-I: a novel trypsin inhibitor from Jatropha curcas seed cake with potential for bacterial infection treatment

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Jatropha curcas seed cake is a low-value by-product resulting from biodiesel production. The seed cake is highly toxic, but it has great potential for biotechnology applications as it is a repository of biomolecules that could be important in agriculture, medicine, and industry. To explore this potential, a novel trypsin inhibitor called JcTI-I was purified by fractionation of the crude extract with trichloroacetic acid (2.5%, v/v) followed by affinity chromatography (Trypsin-Sepharose 4B) and molecular exclusion (Sephadex G-200). Non-reducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis and gel filtration showed that JcTI-I has approximately 20.0 kDa. Mass spectrometry analysis revealed that the intact molecular mass of JcTI-I is 10.252 kDa. Moreover, JcTI-I is a glycoprotein with 6.4% (w/w) carbohydrates, pI of 6.6, N-terminal sequence similarity around 60% to plant albumins and high stability to heat, pH, and salinity. JcTI-I presented antibacterial activity against the human pathogenic bacteria Salmonella enterica subspecies enterica serovar choleraesuis and Staphylococcus aureus, with minimum inhibitory concentration less than 5 μg/mL. Furthermore, JcTI-I did have inhibitory activity against the serine proteases from the tested bacteria. Otherwise, no hemolytic activity of human erythrocytes and signs of acute toxicity to mice were observed for JcTI-I. The results demonstrate the benefits of J. curcas seed cake as a source of trypsin inhibitor with potential for biotechnological application as a new antimicrobial agent against human pathogenic bacteria.

Keywords: Jatropha curcas, seed cake, serine protease inhibitor, trypsin inhibitor, bacterial infections, antimicrobial agent

INTRODUCTION
Hospital-acquired infection is the major cause of death in critically ill patients, both in developing and developed countries (Ramsamy et al., 2013). These infections are a challenge to patient safety because the causal agents have developed antibiotic-resistance, thus limiting treatment options (Mulvey and Simor, 2009; Becker et al., 2012). For example, both the methicillin-resistant Staphylococcus aureus (MRSA) and multidrug-resistant Salmonella enterica are the principal multidrug resistant bacterial pathogens that cause serious community and hospital-acquired infections, responsible for high annual health care costs and psychological stress associated with social stigma (Cosgrove et al., 2003; Brydon et al., 2009; Broughton et al., 2010). Therefore, effective new therapeutic agents with novel mechanisms of action for treatment of infections caused by multidrug resistant bacteria are urgently needed (Hughes et al., 2012). In this context, as plants have numerous therapeutic compounds they constitute natural targets from which new antibacterial drugs with high efficacy and less toxicity can be developed to treat infectious diseases (Ngo et al., 2013). Indeed, biologically active compounds from plant resources have been extracted from different species (Saxoiva, 2012).

Jatropha curcas, also known as physic nut, is a shrub belonging to the Euphorbiaceae family. This species is highly adaptable to adverse conditions and is resistant to many pests and pathogens (Debnath and Bisen, 2008; Sabandar et al., 2013). In addition, J. curcas seeds constitute an oil-rich plant source from which biodiesel is produced (Rashid et al., 2010). After oil extraction by screw press, the remaining seed cake is highly toxic to a number of animal species, probably due to the presence of phorbol esters and curcin, a type-I ribosome inactivating protein (Goel et al., 2007; Zhao et al., 2012). Protease inhibitor, lectin, and phytate are also present in high amounts (Saetee and Suntornsup, 2011). Nevertheless, these compounds could be isolated and characterized to exploit their possible medicinal applications, as it was suggested that the J. curcas seed cake could be utilized as a source of antibacterial and antifungal agents (Sundari and Selvaraj, 2011).

Among these various molecules present in J. curcas seed cake, the protease inhibitors could be a potentially novel class of antimicrobial agents, as they specifically inhibit the catalytic action of enzymes by formation of stoichiometric complex with the target enzymes, blocking or altering its active site (Kim et al., 2009; Volpacciola et al., 2011). In fact, protease inhibitors are found to be involved in various important physiological functions like regulators of endogenous proteases and defense mechanism (Bhattacharjee et al., 2012). However, protease inhibitors have also received new interest due to their biological properties with...
potential for use as clinical agents. Of importance in the context of seeking plant protease inhibitors as novel therapeutic agents is that Xb-KTI, a Kunitz trypsin inhibitor present in Xanthosoma blando corms with bactericidal activity ( Lima et al., 2011). Similarly, the fistulin, a naturally occurring inhibitor of serine protease present in Cassia fistula leaves, showed to be very active against several pathogenic bacterial strains, namely, Staphylococcus aureus, Escherichia coli, Bacillus subtilis, and Klebsiella pneumoniae, and its efficacy was comparable to the standard drug, streptomycin sulfate (Arulpendri and Sangeetha, 2012).

To the best of our knowledge, a trypsin inhibitor from J. curcas seed cake has never been isolated neither its antibacterial activity tested yet. Thus, the present paper describes the purification and physicochemical characterization of a novel trypsin inhibitor from J. curcas seed cake, designated JcTI-I. Additionally, to gain better insights on the biological activity of this protein and to devise future use as a new therapeutic drug, its inhibitory activity against the growth of the human pathogen bacteria Staphylococcus aureus, pseudomonas aeruginosa, and Salmonella enterica, as well as the ability of JcTI-I to inhibit the pancreatic proteases were evaluated.

MATERIALS AND METHODS

MATERIALS

Jatropha curcas seed cake was obtained from Instituto Fazenda Tamanduá (Paraíba, Brazil), ground in a coffee grinder and passed through a 1-mm-mesh screen. The resulting flour was treated with n-hexane (1:5, v/v) to remove the remaining oil left after biodiesel extraction. Defatted flour was stored in air-tight containers at 4°C until analysis. The bacteria Salmonella enterica subspecies enterica serovar cholerasuis (ATCC 10708), Bacillus subtilis subsp. spizizenii (ATCC 6633), Pseudomonas aeruginosa (ATCC 25619), and Staphylococcus aureus (ATCC 29233) were obtained from the Department of Biology (UFU), Goiás, Brazil. Swine mice (Mus musculus), 20–25 g, were from the animal house at UFC. Anococin, bovine pancreatic trypsin, bovine pancreatic chymotrypsin, bovine serum albumin (BSA), soybean trypsin inhibitor (SRTI), Na-benzoyl-D,L-arginina-p-nitroanilida (BAPNA), 4-(dimethylamino)cinnamaldehyde (DMACA), ethylene-diaminetetraacetic acid (EDTA), phenylmethylsulfonil fluoride (PMSF), L-cysteine and Na-benzoyl-D,L-arginine-4-nitroanilida hydrochloride (BAPNA), sodium dodecyl sulfate (SDS), molar mass markers, acrylamide, bis-acrylamide, dithiothreitol (DTT) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Chromatographic matrices were from GE Healthcare. All other chemicals and reagents were of analytical grade.

PROTEIN DETERMINATION

The method described by Bradford (1976) was used with BSA as standard. Absorbance at 280 nm was also used to detect the presence of protein in the chromatographic eluates.

TRYPsin INHIBITION ASSAY

Trypsin inhibitory activity against bovine trypsin (EC 3.4.21.4) was performed as described by Erlanger et al. (1961) using the chromogenic substrate BAPNA. The enzyme concentration used was established based on a dependent concentration assay and was that producing an absorbance between 0.25 and 0.30 in the enzymatic assay and reaction rate corresponding to ½ Vmax. The inhibitory assay mixture consisted of 100 μL of crude extract (4.74 mg/mL) or the purified inhibitor (0.03 mg/mL) dissolved in distilled water, incubated with 10 μL of trypsin (0.3 mg/mL in 10−3 M HCl) and 690 μL of 0.05 M Tris–HCl/0.02 M CaCl2, pH 7.5, at 37°C for 10 min. Next, 500 μL of 1.25 × 10−3 M BAPNA dissolved in the above buffer were added and the mixture further incubated for 15 min, 37°C. Reaction was stopped by addition of 120 μL of 30% (v/v) acetic acid. The enzymatic activities in the absence and presence of the inhibitor were evaluated by p-nitroanilide release from BAPNA measured at 410 nm using a Biochrom Libra S-12 spectrophotometer. One trypsin inhibitory activity unit (TIU) was defined as the decrease in 0.01 U of absorbance per 15 min assay, at 37°C. Appropriate blanks for the enzyme, inhibitor and the substrate were also included in the assay along with the test samples.

PREPARATION OF THE PROTEIN EXTRACT

Defatted flour was extracted with 0.1 M borate buffer, pH 10.0, in a proportion of 1.0 g of meal to 10.0 mL of buffer for 2 h under sonication, at 4°C. Next the suspension was maintained under constant stirring for 2 h at 4°C and filtered through cheesecloth. The filtrate was centrifuged at 10,000 × g, 4°C, 30 min and the clear supernatant, denoted crude extract, was dialyzed (cut-off 12 kDa) against 0.050 M sodium phosphate buffer, pH 7.5. The protein content and trypsin inhibitory activity of this dialyzed extract were determined and it was further used for purification of the trypsin inhibitor as described below.

PURIFICATION OF J. curcas TRYPsin INHIBITOR

The crude extract prepared as described in Section “protein Determination” was fractionated by precipitation with 2.5% (v/v) trichloroacetic acid (TCA) final concentration, at 4°C and centrifuged at 14,000 × g, 4°C, 30 min. The clear supernatant obtained was dialyzed exhaustively against water (Milli-Q grade), lyophilized, and assayed for antiprotease activity. The TCA fraction (30 mg) was dissolved in 0.050 M sodium phosphate buffer/0.2 M NaCl, pH 7.5 and applied to a trypsin-Sepharose 4B column (11.5 cm × 2.2 cm) equilibrated with the above buffer. After complete removal of the non-retained proteins with the equilibrating buffer, the proteins bound to the immobilized trypsin were eluted with 0.1 M HCl, dialyzed exhaustively against water (Milli-Q grade) and lyophilized. This material (2 mg) was loaded on a Sephacryl S-200 column connected to an ÄKTA-Prime System (GE Health-care) previously equilibrated and eluted with 0.050 M sodium phosphate buffer/0.2 M NaCl, pH 7.5. Fractions (1 mL) were eluted at the flow rate of 0.5 mL/min and the protein fractions obtained evaluated for trypsin inhibitory activity as described before. The purified trypsin inhibitor was named JcTI-I (J. curcas Trypsin Inhibitor I).

CHARACTERIZATION OF JcTI-I

Molecular mass determination

The apparent molecular mass of JcTI-I was determined by denaturing electrophoresis [SDS-polyacrylamide gel electrophoresis

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Analysis was performed using a nanoelectrospray ionization tandem mass spectrometry (ESI–LC–MS/MS), using the same equipment as described above, coupled with a nano-HDMS Acquity UPLC instrument (Waters Co., Milford, MA, USA). The reduced and alkylated JcTI-I was subjected to data-dependent acquisition (DDA) methodology, where the three top peaks were subjected to an ÄKTA-Prime System (GE Healthcare) and equilibrated with 0.25 M sodium phosphate buffer, pH 6.5 kDa). The intact mass spectra was acquired in a Synapt G1 HDMS Acquity UPLC instrument (Waters Co., Milford, MA, USA) programmed with a RF offset (MS profile) adjusted such that the LC/MS data were effectively acquired from 0.07 to 3,000, which allowed to obtain multiply charged mass ions. This was evaluated according to Klomklao et al. (2011). JcTI-I activity was determined and reported as the percentage of the residual activity after incubation at various pH values for 30 min at 25°C. The buffers used were: 0.05 M glycine–HCl, pH 2.2; 0.05 M sodium acetate, pH 5.2; and 0.1 M sodium borate, pH 10.8. The stability of JcTI-I to salinity was tested by incubating the trypsin inhibitor in NaCl solutions ranging from 0% up to 3% (m/v) concentration, at 25°C for 30 min. After treatment the inhibitory activity against trypsin was determined as previously described and the residual inhibitory activity reported.

**Enzyme specificity**

The papain inhibitory assay was performed as described by Abe et al. (1992), using BANA as substrate. The enzyme concentration used was established based on a dependent concentration assay and was that producing an absorbance between 0.25 and 0.30 in the enzymatic assay and reaction rate corresponding to 1/3 Vmax.

The neutral sugar content of JcTI-I was estimated by the method of Dubois et al. (1956) using galactose as standard. To 250 μL of JcTI-I (0.20 mg/mL), 230 μL of phenol 5% (v/v) and 1.25 mL of concentrated sulfuric acid were added. The mixture was stirred and left at 25°C for 30 min. Readings were taken at 490 nm. The carbohydrate content was estimated based on a calibration curve built with different concentrations of galactose. The value is expressed in percentage (% of carbohydrate per protein mass. To confirm the presence of covalently bound carbohydrate in JcTI-I a specific staining of the protein bands after SDS-PAGE was carried out (Zacharius et al., 1969). Briefly, after the electrophoresis run the gel was fixed in a solution of 7.5% (v/v) acetic acid for 2 h, followed by incubation in a solution of 0.2% (v/v) periodic acid, at 4°C for 45 min and incubation with the Schiff reagent at 4°C for further 45 min. The glycoprotein nature of the band was revealed by immersion of the gel in a solution of 0.5% (m/v) potassium meta bismuthate in 0.05 M HCl.

**N-terminal sequence analysis**

This was established in a Shimadzu PPSQ-10 Automated Protein Sequencer performing Edman degradation. Phenylthiohydantoin (PTH) amino acids were detected at 269 nm after separation on a reversed phase C18 column (4.6 mm × 25 mm) under isocratic conditions, according to the manufacturer's instructions. Percent sequence identity with trypsin inhibitors was searched for automatic alignment, performed using the NCBI-BLAST search system (Altschul et al., 1990).

This was performed using Masslynx 4.1 software (Waters Co., Milford, MA, USA) and the searches made with the assumption that there was a maximum of one missed trypsin cleavage, peptides were monoisotopic, methionine residues were partially oxidized and cysteine completely carbamidomethylated.
mixture was incubated for 10 min at 37°C. The reaction was initiated by addition of 200 μL 0.001 × 10⁻³ M BANA, prepared in 1% (v/v) dimethyl sulfoxide (DMSO) and 0.025 M sodium phosphate buffer, pH 6.0. After 20 min at 37°C, the reaction was stopped by addition of 500 μL 2% (v/v) HCl in 95% (v/v) ethanol. The color product was developed by adding 500 μL of 0.06% (v/v) DMAA in ethanol and the absorbance was measured at 540 nm. The chymotrypsin inhibitory activity was measured by the method of Elgar et al. (1961), using azocasein as substrate. Twenty microliters of chymotrypsin (0.1 mg/mL in 2.5 × 10⁻³ M HCl) was pre-incubated with JcTI-I (0.2 mg/mL) and 380 μL of 0.05 M Tris-HCl pH 7.5, for 15 min, 37°C. Next, 1% (m/v) azocasein was added to the mixture and incubated for 30 min. Reaction was stopped by addition of 300 μL 20% (v/v) TCA. After centrifugation (10,000 × g, 10 min, 25°C), aliquots were withdrawn from the supernatants and added to 2 M NaOH, in an appropriate proportion, and the absorbance taken at 440 nm.

**IC⁵₀ value and kinetic studies**

The amount of JcTI-I needed for 50% inhibition of trypsin activity was determined as previously described in Section “Trypsin Inhibition Assay”, but using the inhibitor in the concentration range of 1.0 × 10⁻¹ to 2.0 × 10⁻⁷ M. Kinetic studies of protease inhibition by JcTI-I were conducted according to Bigna et al. (2011), with minor modifications, using bovine trypsin (molecular weight 23.3 kDa) and different concentrations of BAPNA (8.0 × 10⁻⁷ to 1.6 × 10⁻³ M). To calculate the kinetic parameters, JcTI-I at 5 × 10⁻² and 10 × 10⁻⁷ M concentrations and 8.0 × 10⁻⁴ M trypsin were previously incubated in 0.05 M Tris–HCl 0.02 M CaCl₂, pH 7.5, for 10 min, at 37°C, to reach the assay temperature. Then, 100 μL fractions of JcTI-I were added to 10 μL of the trypsin solution and further incubated for 10 min, at 37°C. Next, both the trypsin solution and trypsin + JcTI-I mixture were added separately to the BAPNA solutions and incubated at 37°C, for 15 min. Reaction was stopped by addition of 120 μL of 30% (v/v) acetic acid. The release of p-nitroanilide by the action of trypsin on BAPNA was measured at 410 nm as before. The velocity of enzyme reaction (v) was determined from progress curves using different BAPNA concentrations on the basis of product concentrations measured at 410 nm at a fixed time of a reaction. To calculate $K_m$ (Michaelis–Menten constant) and $V_{max}$ (maximal rate) of the reaction and to study the pattern of inhibition, data were plotted as a function of BAPNA concentration using the usual non-linear curve fitting of Michaelis–Menten and linear Lineweaver–Burk, respectively. A secondary plot of $V_{max}$/versus JcTI-I concentrations was also drawn and the X-intercept used to calculate the dissociation constant ($K_i$, Cornish-Bowden, 1995).

**Antimicrobial activity**

in vitro antibacterial activity

The antimicrobial activity (Hancock, 2000) of JcTI-I was tested in vitro against Salmonella enterica, Staphylococcus aureus, Bacillus subtilis, and Pseudomonas aeruginosa. The pathogenic bacteria were grown to mid-logarithm phase in 5 mL of Mueller–Hinton broth medium, at 37°C. JcTI-I, at a 500 μg/mL final concentration, was prepared in 0.25 M sodium phosphate buffer, pH 7.5, and sterilized using a 0.22 μm membrane, was incubated in 96-well flat microplates (Nunc) with 100 μL of each bacterial suspension (10²–10⁶ CFU/mL) dissolved in the growth broth for 4 h at 37°C. Bacterial growth was monitored at 630 nm, every hour within the incubation period, using an automated microplate reader (BioTekElx800). The cell growth of both bacteria in the absence of JcTI-I was monitored as a blank control. Experiments were run in triplicate. To determine the minimum inhibitory concentration (MIC), JcTI-I, at 5–500 μg/mL concentration, was dissolved in 0.25 M sodium phosphate buffer, pH 7.5 and 100 μL incubated with equal volume of the bacterial cell suspension (10²–10⁶ CFU) in wells of a 96-well polypolyethylene plate. The plates were kept for 4 h at 37°C and absorbance readings recorded at 630 nm every hour.

Activity of JcTI-I against the Salmonella enterica and Staphylococcus aureus proteases

The capacity of JcTI-I to inhibit the secreted and endogenous bacterial proteases was done following the methodology described by Lima et al. (2011) with minor modifications. Salmonella enterica and Staphylococcus aureus were cultured in 5 mL of Mueller–Hinton broth overnight, at 37°C, and subcultured in 300 mL of Mueller–Hinton broth, at 37°C and 240 rpm, until the log phase was reached, as measured at 630 nm (Bio-TekElx880 reader). The bacterial cell cultures were collected at the late exponential growth phase by centrifugation at 4,000 × g, for 15 min, at 4°C and the pellet resuspended in the protease extraction solution [HCl 0.1% (v/v) + 5.0 × 10⁻⁵ M Tris + 5.0 × 10⁻⁷ M CaCl₂ + 0.1% (v/v) Triton X-100, pH 7.5] and incubated in ice bath for 35 min. Freezing and thawing took place three times, followed by centrifugation at 4,000 × g, for 30 min, at 4°C. The supernatants obtained, containing the protease from Salmonella enterica and Staphylococcus aureus, were pooled separately for every bacterium species and the serine protease activity as well as the inhibitory activity of JcTI-I assayed, according to the method previously described, using azocasein as substrate, bovine trypsin and PMSF as serine protease control inhibitor.

**Hemolytic activity assay**

The hemolytic assay, performed in triplicate, was carried out as previously described (Kim et al., 2006), with minor modifications. Human red blood cells were collected in the presence of heparin, washed three times with phosphate-buffered saline (PBS; 3.5 × 10⁻³ M phosphate buffer containing 0.15 M NaCl, pH 7.0) and recovered by centrifugation in the same buffer. The hemolytic activity of JcTI-I at 5–500 μg/mL concentration was evaluated by measuring the release of hemoglobin from fresh human erythrocytes. Aliquots (25 μL) of a 2.5% suspension of red blood cells were transferred to 96-well plates and incubated with 25 μL of JcTI-I for 30 min, at 37°C, followed by centrifugation at 5,000 × g, for 5 min. Hemolysis (%) was determined by measuring the supernatant absorbance at 540 nm. PBS (0.05 M, pH 7.4) and 0.1% (v/v) Triton X-100 were used, respectively, as negative (0% hemolysis) and positive (100% hemolysis) controls.
TOXICITY ASSAY
The toxicity assay was reviewed and approved by the Animal Ethics Committee (CEPA) of UFC, Brazil, and realized according to the methodology described by Vasconcelos et al. (1994). Toxic activity was defined as mortality observed in Swiss mice within 24 h after intraperitoneal injections of JcTI-I at varied concentrations.

STATISTICAL ANALYSIS
The results are expressed as the mean ± SEM. The statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Tukey’s multiple comparison tests. The results were considered to be significant at $P < 0.05$.

RESULTS

PURIFICATION OF JcTI-I
Purification of JcTI-I encompassed TCA precipitation of the J. curcas protein extract followed by two additional chromatography steps. TCA, at 2.5% (v/v) final concentration, precipitated the proteins with a recovery of up to 35.6% and 29.0-fold purification of the trypsin inhibitor compared to the protein extract (Table 1). This TCA precipitated fraction was chromatographed on a trypsin-Sepharose 4B affinity column and the adsorbed proteins, eluted with 0.1 M HCl, concentrated the inhibitory activity that was specific for trypsin (Figure 1A), indicating that this chromatography step was important to exclude other protease inhibitors also present in the TCA precipitated fraction, as for papain (data not shown). At this stage, the trypsin inhibitor was purified to 331.5-fold with a protein yield of 1.8% and specific activity of $6.63 \times 10^{-3}$ TIU/mg protein (Table 1). The fraction obtained from the trypsin-Sepharose 4B affinity chromatography displaying trypsin inhibitory activity was composed of multiple protein bands as evaluated by SDS-PAGE (Figure 1A, insert: lane 5). Therefore, this fraction was further purified on a Sephacryl S-200 column (Figure 1B). The eluted protein present in the major peak showed a specific activity of $28.35 \times 10^{-3}$ TIU/mg protein, 1417.5-fold purification, but a low protein yield of 1.2%. Nonetheless, it was homogenous by SDS-PAGE as it presented a unique protein band with a relative molecular mass around of 20.0 kDa (Figure 1B, insert: lane 2). This purified protein is a trypsin inhibitor of J. curcas seed cake that was denominated JcTI-I thereafter.

CHARACTERIZATION OF JcTI-I
Molecular mass determination, $p$-value, carbohydrate content, and $N$-terminal sequence
Native molecular mass of JcTI-I was assessed by gel filtration chromatography on Sephacryl S-200 column. A molecular mass of 20.2 kDa was calculated for the inhibitor dissolved in 0.05 M

Table 1 | Purification steps of a trypsin inhibitor from J. curcas cake.

| Steps                  | Total protein$^a$ (mg) | Total activity$^b$ (TIU $\times 10^{-3}$) | Specificity activity (TIU/mg protein $\times 10^{-3}$) | Yield$^c$ (%) | Purification index$^d$ |
|------------------------|------------------------|----------------------------------------|---------------------------------------------------|--------------|-----------------------|
| Crude extract          | 165.6                  | 4.2                                    | 0.02                                              | 100          | 1.0                   |
| F$_{2.5}$ (TCA)        | 59.0                   | 34.4                                   | 0.58                                              | 35.6         | 29.0                  |
| Trypsin-Sepharose 4B   | 3.0                    | 19.9                                   | 6.63                                              | 1.8          | 331.5                 |
| Sephacryl S-200        | 2.0                    | 56.7                                   | 28.35                                             | 1.2          | 1417.5                |

Results are presented as mean values of three replications.
$^a$The total amount of protein recovered from 1 g of defatted flour from J. curcas seed cake.
$^b$One trypsin inhibitory activity unit (TIU) was defined as the decrease in 0.01 U of absorbance per 15 min assay, at 37°C.
$^c$The recovery of protein at each purification step (crude extract, 100%).
$^d$Purification index is calculated as the ratio between the specificity activity obtained at each purification step and that of the crude extract taken as 1.0.

Figure 1 | JcTI-I purification. (A) Affinity-chromatography (Trypsin-Sepharose 4B column). Inset: SDS-PAGE: (1) molar mass markers; (2) crude extract; (3) 2.5% TCA fraction; (4) unbound fractions; (5) bound fractions. (B) Size exclusion chromatography (Sephacryl S-200 column) of JcTI-I purified after chromatography on Trypsin-Sepharose 4B column. Inset: SDS-PAGE: (1) molar mass markers; (2) and 3. JcTI-I (5 μg) in the absence and presence of 1% β-mercaptoethanol, respectively; (4) JcTI-I (5 μg) stained with Schiff’s reagent.
sodium phosphate buffer, pH 7.5, containing 0.2 M NaCl. This value is very similar compared to the molecular mass obtained by SDS-PAGE in the absence of reducing agents (Figure 1B, insert: lane 2). However, ESI mass spectrometry under native conditions, with JcTI-I dissolved in water/acetonitrile (1:1, v/v), revealed a 10.252 kDa polypeptide (Figure 2A) which is very close to the molecular mass of JcTI-I found under denaturing condition in the presence of β-mercaptoethanol (Figures 1B, insert: lane 3) and after two-dimensional (2D) gel electrophoresis that also showed a pI of 6.6 (data not shown) for the purified inhibitor. DTT reduced and alkylated JcTI-I produced two derived peptides of 7.133 and 3.124 kDa, after MS/MS analysis, suggesting that these polypeptides are linked by disulfide bond (Figures 2B,C, respectively). Moreover, JcTI-I has 6.4% covalently linked carbohydrate (Figure 1B, insert: lane 4) and an N-terminal sequence of VRDICKKEAERRDLSSCENYITQRRGY (Table 2). This sequence was confirmed after mass spectrometry analysis of JcTI-I (Table 3). Alignment of this N-terminal sequence with known sequences in the NCBI non-redundant database showed 68 and 58% similarity with an allergenic 2S albumin from J. curcas seeds and with an albumin 2S from Ricinus communis, respectively. In addition, other peptide sequences were obtained similar to 2S albumins, particularly from R. communis (Table 3). However, no similarity was found between the N-terminal sequence of JcTI-I generated by Edman degradation and the peptide sequences obtained by MS/MS analysis with known members of the protease inhibitor family.

**Thermal and pH stability**

Heating of JcTI-I at 90°C for up to 20 min induced a slight increased in the inhibitory activity upon trypsin as compared to 70% trypsin inhibition of the native inhibitor (Figure 3A). Afterward the heat treatment for up to 120 min had little consequence on the inhibitory effect of JcTI-I as it was reduced only to around 4–5% of that of the untreated inhibitor. Incubation of JcTI-I at the pH range of 2.0–10.0 (Figure 3B) had overall a slight inducing effect (10%) on inhibition (Figure 3B), except at pH 5.0 that maintained 70% trypsin inhibition. Incubation of JcTI-I with different concentrations of salt (0–3%) showed that the protein retained approximately its original activity up to 2.0% NaCl concentration (Figure 3C). However, at 2.5% NaCl concentration an 11.6% reduction in the inhibitory activity was noticed (Figure 3C), whereas at 3.0% the trypsin inhibition was 10% higher than the 70% inhibition induced by the untreated inhibitor.

**Enzyme specificity**

JcTI-I was specific for inhibiting bovine trypsin, but did not inhibit significantly chymotrypsin and papain.

**IC₅₀ value and kinetic parameters**

The data obtained for the studies conducted on protease–protease inhibitor interaction is depicted in Figure 4. The amount of inhibitor needed for 50% trypsin inhibition (IC₅₀) was 1.25 × 10⁻⁶ M. Moreover, it is calculated that the stoichiometry of trypsin–protease inhibitor interaction is 1:1.7. Kinetic studies of inhibition of trypsin by JcTI-I showed that plots of a fixed concentration of trypsin (8.0 × 10⁻⁶ M) preincubated with the buffer alone (control) and with 0.5 and 1.0 × 10⁻⁶ M of JcTI-I produced different slopes of 1/v versus 1/[s] (Figure 5). These data indicated a non-competitive mechanism of action of JcTI-I as lines are converging to the same point on the 1/[S] axis where Km is unchanged, whereas Vmax is reduced. Furthermore, calculated Ki (Figure 5, insert) was on average 0.24 × 10⁻⁶ M, indicating that inhibition of the trypsin hydrolysis by JcTI-I occurred at very low concentration characterizing a very potent inhibitor.
Table 3 | Peptide sequences of JcTI-I identified after data-dependent acquisition (DDA) analysis.

| m/z       | Charge | Delta (ppm) | Sequence                      | Modifications                                      |
|-----------|--------|-------------|-------------------------------|---------------------------------------------------|
| 821.5901  | 2      | 4.091931    | (PR)DLSCEVITORGPR             | Carboxymethyl C (6)                                |
| 743.3438  | 2      | 10.359877   | (RIOLSSCENITQPR)              | Carboxymethyl C (6)                                |
| 424.7177  | 2      | −2.8809798  | (K)ELSAIRCIC                  | Carboxymethyl C (6)                                |
| 646.3268  | 2      | 7.503016    | (PCESHYLIKEK)                 | Carboxymethyl C (1)                                |
| 734.3472  | 2      | 2.6834285   | (KI)GLEEVGISEDGARMH           |                                                   |

*Results of a BLAST search.

Antimicrobial activity of JcTI-I

JcTI-I did not affect the growth of Bacillus subtilis and Pseudomonas aeruginosa, even at a concentration of 500 μg/mL (data not shown). However, the protein inhibited the growth of Staphylococcus aureus and almost 100% inhibition of the proteases extracted from Salmonella enterica at all concentrations tested when compared to controls. The MIC was calculated as 3 μg/mL for both bacteria (Figures 6A,B). Moreover, JcTI-I caused 84.6% and 100% inhibition of the proteases extracted from Staphylococcus aureus and Salmonella enterica, respectively, values similar to that found for protein sparing modified fast (PSMF; Figure 7).

Assessment of hemolytic activity of JcTI-I

JcTI-I did not promote hemolysis of human erythrocyte under the experimental conditions tested (Figure 8).

Assessment of toxic activity of JcTI-I

JcTI-I did not display toxic activity to mice, even at a concentration 100 times greater than the MIC for Staphylococcus aureus and Salmonella enterica.

Discussion

Purification of a novel trypsin inhibitor (JcTI-I) from J. curcas seeds cake was achieved by TCA precipitation of the protein extract followed by two chromatographic steps. JcTI-I is a 10.252 kDa neutral glycosylated protein, with a pI of 6.6 and 6.4% of carbohydrate. Both the molecular mass and pI are similar to other seed trypsin inhibitors previously characterized as, for example, the 10.0 kDa trypsin inhibitor from Zia mays (Baker et al., 2009) and that purified from Phaseolus limensis seeds, with pI of 7.6 (Wang and Rao, 2010).

Analyzing the results of both SDS-PAGE and MS/MS, it appears that the 10.252 kDa JcTI-I molecule is composed of a 7.133 and 3.124 kDa polypeptides joined together by at least one disulfide bridge and that DTT + iodoacetamide treatments, but not β-mercaptoethanol alone, were able of irreversibly splitting the molecule in the small and large subunits. The results of size exclusion chromatography of JcTI-I on Sephacryl S-200 gave a 20.2 kDa molecule. It is plausible that under such mild conditions in which JcTI-I was dissolved in 0.050 M sodium phosphate buffer containing 0.2 M NaCl, pH 7.5, the 10.252 kDa monomer associated to form a 20.2 kDa dimer, which does not occur in the presence of acetonitrile in the MS/MS analysis.

Alignments of JcTI-I sequence with other protein sequences showed that the highest similarity was verified with Jat c 1, a J. curcas allergen that belongs to the 2S albumin family (Maciel et al., 2009), but not with known protease inhibitor sequences.

Several trypsin inhibitors show sequence similarities with the 2S albumin-like proteins, as two barley Bowman–Birk type trypsin inhibitor isoforms and the trypsin inhibitor of Brassica juncea seeds (Terra et al., 1993; Mandal et al., 2002). Therefore, JcTI-I might be a novel trypsin inhibitor that belongs to the 2S albumin family.

Protease inhibitors exhibit a considerable stability to high temperatures and to large pH variations (Bhattacharyya et al., 2006; Bijuna et al., 2011). JcTI-I has also these common properties as it was active and stable after incubation at 90°C for 60 min and in the pH range 2.0–10.0, in agreement with other plant protease inhibitors. Moreover, JcTI-I was stable to high salt concentrations as it retained full activity after treatment with up to 2% NaCl. The trypsin inhibitor purified from adzuki bean (Vigna angularis) seeds was also stable when
JcTI-I is a non-competitive trypsin inhibitor similar to other trypsin inhibitors, such as AETI, APTI, and TTI from the seeds of *Archidendron ellipticum* (Bhattacharyya et al., 2006), *Adenanthera pavonina* (Macedo et al., 2004), and *Tamarindus indica* (Arasjo et al., 2005), respectively. The IC50 of JcTI-I for trypsin was $1.25 \times 10^{-6}$ M, indicating that it was more potent than EvTI, the trypsin inhibitor from *Erythrina velutina* seeds ($IC50 = 2.2 \times 10^{-6}$ M; Machado et al., 2013) and less than SSTI2, the trypsin inhibitor from *Sapindus saponaria* seeds.
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FIGURE 6 | Effect of JcTI-I at different concentrations on the growth of Staphylococcus aureus (A), and Salmonella enterica (B). Control: 0.05 M sodium phosphate buffer (pH 7.5). The minimal inhibitory concentration (MIC) of JcTI-I was determined based on these data. Assay was done in triplicate for every inhibitor concentration. The standard deviation was less than 10%.

FIGURE 7 | Inhibitory effect (%) of JcTI-I on the proteases extracted from Staphylococcus aureus and Salmonella enterica. Asterisks represent significant difference (P < 0.05) in comparison to control (0.25 M sodium phosphate buffer, pH 7.5). The experiments were run in triplicate and each value is presented as mean ± standard deviation.

FIGURE 8 | Assessment of the hemolytic effect of JcTI-I on human erythrocytes. A suspension of washed human erythrocytes was incubated with various concentrations of JcTI-I (-▲-). Values (OD540) were normalized against 100% lysed erythrocytes (0.1% Triton X-100) (-•-). Control was the red cells incubated with 0.05 M PBS, pH 7.4 (-■-). Each point represents the mean of three estimates. The standard deviation was less than 10%.

Resistance of some strains of Gram-positive and Gram-negative bacteria to conventional antibiotics has increased dramatically caused or induced by the widespread misuse and overuse of antibiotics and represents a serious threat to public health worldwide. Therefore, discovery and/or development of alternative, non-conventional drugs with activity against most resistant bacteria for infection control are of paramount importance (Lima et al., 2011).

J. curcas is a plant traditionally used for medicinal purposes and its antimicrobial potential has been previously reported. Indeed, extracts from leaves, root barks, latex, and various fruit parts of J. curcas showed inhibitory activity against E. coli, Staphylococcus aureus, Pseudomonas aeruginosa, and Bacillus subtilis (Arekemase et al., 2011; Nyembo et al., 2012; Rachana et al., 2012). However, few reports are related to the antimicrobial activity of compounds present in the seed cake of J. curcas, although its potential use as a source of antibacterial agents has...
been suggested (Sripriya et al., 2010; Sundari and Selvaraj, 2011). The seed cake of *J. curcas* represents an attractive raw material to extract novel antibacterial drugs because it is produced in large scale as a waste material after oil extraction to produce biodiesel. It is estimated that 1 ton per day of *J. curcas* extracted oil generates, approximately, 3 tn of seed cake (Sripriya et al., 2010).

The use of protease inhibitors as novel therapeutics has previously been proposed (Bhattacharjee et al., 2012; Machado et al., 2013). Therefore, take into consideration the huge amount of *J. curcas* seed cake generated, from which JcTI-I was purified, the hypothesis that it could behave as a natural drug against human pathogens was also tested. This was experimentally confirmed as JcTI-I behaved as a potent inhibitor of *Staphylococcus aureus* and *Salmonella enterica* growth. The MIC of JcTI-I for both bacteria was 5 μg/mL. This concentration is sixfold lower compared with that found for a potato inhibitor effective against various bacteria, including *Staphylococcus aureus* with a MIC around 30 μg/mL (Kim et al., 2006), about 50-fold lower than that of Xd-KTI, a kunitz protease inhibitor from *X. blandum* seeds, active against *Salmonella typhimurium*, with a MIC of about 256 μg/mL (Lima et al., 2011) and about 25-fold lower than that of clonacillin, a β-lactam antibiotic used as therapeutic drug against MRSA with a MIC around 128 μg/mL (Islam et al., 2008). According to Hancock (2000), the best protein/peptide candidates for antibacterial drugs have MICs ranging from 1 to 8 μg/mL. Based on these results, JcTI-I can be considered as a potent inhibitor of bacterial growth and could be explored as an antibacterial protein to help bring down the 10–20% mortality caused by *Staphylococcus aureus* (van Hal et al., 2012).

Serine proteases have been implicated in the virulence of some bacterial strains (Speranskaya et al., 2006; Tripathi et al., 2011). Therefore, substances that can interfere with the proteolytic activity of these enzymes, like protease inhibitors, could be an effective strategy to combat bacterial infections (González-Lamothe et al., 2009). JcTI-I did have inhibitory activity against the serine proteases from the tested bacteria. It caused about 85 and 100% inhibition of the proteases extracted from *Staphylococcus aureus* and *Salmonella enterica*, respectively, values compared to those achieved by PSM-A, a synthetic inhibitor. These results are similar to that reported by Lima et al. (2011) who found that Xd-KTI caused about 80% inhibition of the serine proteases from *Salmonella typhimurium*.

To assess whether JcTI-I promotes lysis of eukaryotic cells, this protease inhibitor was incubated with human erythrocytes up to the concentration of 500 μg/mL, about 10-fold higher than the MIC of JcTI-I (5 μg/mL) for both bacteria. Even at such concentration, JcTI-I does not lysed the red cells. This result suggests that the mode of action of JcTI-I is not by disrupting cell membranes and it does not have toxic effects toward mammalian cells. In addition, JcTI-I was not toxic to mice at a concentration very much higher than the MIC for *Staphylococcus aureus* and *Salmonella enterica*.

In summary, we have isolated, purified, and characterized a novel trypsin inhibitor from *J. curcas* seed cake, named JcTI-I, which possesses a potent activity against the human pathogenic bacteria *Staphylococcus aureus* and *Salmonella enterica*. The lack of hemolytic activity against human erythrocytes and toxic activity to mice together with resistance to heat treatment, pH high salt concentrations, and putative resistance to proteases make JcTI-I a pharmacologically interesting and valuable drug for the design of a novel antibiotic medicament.

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