

# Chia (*Salvia hispanica* L.) seeds contain a highly stable trypsin inhibitor with potential for bacterial management alone or in drug combination therapy with oxacillin

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## Research Article

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# Abstract

The emergence of antibiotic resistance poses a serious and challenging threat to healthcare systems, making it imperative to discover novel therapeutic options. This work reports the isolation and characterization of a thermostable trypsin inhibitor from chia (*Salvia hispanica* L.) seeds, with antibacterial activity against *Staphylococcus aureus* sensitive and resistant to methicillin. The trypsin inhibitor ShTI was purified from chia seeds through crude extract heat treatment, followed by affinity and reversed-phase chromatography. Tricine-SDS–PAGE revealed a single glycoprotein band of ~11 kDa under nonreducing conditions, confirmed by mass spectrometry analysis (11.558 kDa). ShTI was remarkably stable under high temperatures (100 °C; 120 min.) and a broad pH range (2 – 10; 30 min.). Upon exposure to DTT (0.1 M; 120 min.), ShTI antitrypsin activity was partially lost (~ 38%), indicating the participation of disulfide bridges in its structure. ShTI is a competitive inhibitor ( $K_i = 1.79 \times 10^{-8}$  M;  $IC_{50} = 1.74 \times 10^{-8}$  M) that forms a 1:1 stoichiometry ratio for the ShTI: trypsin complex. ShTI displayed antibacterial activity alone (MICs range from 15.83 to 19.03  $\mu$ M) and in drug combination with oxacillin (FICI range from 0.20 to 0.33) against strains of *S. aureus*, including methicillin-resistant strains. Overproduction of reactive oxygen species and plasma membrane pore formation are involved in the antibacterial action mode of ShTI. Overall, ShTI represents a novel candidate for use as a therapeutic agent for the bacterial management of *S. aureus* infections.

## 1. Introduction

Antimicrobial resistance (AMR) has become one of the most severe public health problems with significant global impacts. Overuse, misuse, and inadequate prescription of antibiotics, among other causes, have been attributed to the burgeoning emergence of multiresistant bacteria, generating the so-called antibiotic resistance crisis [1, 2]. Resistant strains limit the efficacy of antibiotics and are responsible for an increase in infection severity and prolonged therapy duration compared to susceptible strains. Consequently, these pathogens raise healthcare costs and the chances of treatment failures, leading to clinical complications or mortality [3, 4].

Despite the critical situation, this scenario has been accompanied by a low rate of the availability of novel antibiotics [5]. Only twelve new antibacterials have been approved worldwide since 2017 [6]. Economic and regulatory obstacles are among the main factors in the slow antibiotic development pipeline [2]. Alternative therapies are being investigated as a strategy to overcome AMR and replace antibiotics that are becoming obsolete. Approaches involving bacteriophages, synthetic antimicrobial peptides, antibodies, and probiotics have been adopted [7]. In addition, molecules from plant sources have also received considerable attention [8].

Plant protease inhibitors (PPIs) appear to be promising molecules with antimicrobial activity [8, 9]. PPIs are present in different tissues, such as leaves, flowers, fruits, and storage tissues, such as seeds and tubers, representing approximately 1–10% of the total soluble protein content [10, 11]. In addition to their potent antimicrobial activity, the remarkable physicochemical stability (commonly associated with PPIs)

has attracted the attention of the scientific community to those molecules [12]. These features have practical relevance to the pharmaceutical industry. Thus, PPIs have been considered excellent candidates for novel therapeutic agents with antimicrobial activity [12].

*Salvia hispanica* L., popularly known as chia, is an herbaceous annual plant belonging to the Lamiaceae family. Recently, chia seed consumption has increased and gained popularity due to its functional and nutraceutical properties, which greatly benefit human health [13, 14]. The high lipid content (especially alpha-linolenic ( $\omega$ -3) and alpha-linoleic acids ( $\omega$ -6)) is mainly responsible for the increasing demand for chia seeds in the functional food market [15]. Chia seeds also have a high protein content and are considered a promising source of bioactive proteins and peptides with health benefits [13]. Indeed, protein hydrolysates of chia seeds with antimicrobial [16], anti-inflammatory [17], in vitro antioxidant [18], and angiotensin-converting enzyme inhibitory [19] activities have been reported. The isolation of a trypsin inhibitor from chia seeds has also been described [20]. However, its biochemical characterization and bioactive properties have not been investigated.

This study reports the purification and characterization of a trypsin inhibitor from mucilage and fat-free seeds of *S. hispanica* L., *ShTI*, with antibacterial activity. The outcomes will demonstrate a novel candidate with potential application in bacterial management against *Staphylococcus aureus*, including methicillin-resistant strains.

## 2. Materials And Methods

### 2.1. Chia seeds and reagents

*Salvia hispanica* L. seeds were obtained from the local market in Fortaleza, Ce, Brazil, and stored at -20°C until protein extraction and trypsin inhibitor purification. First, chia seed mucilage and fat removal were performed as follows. Chia seeds were soaked in water (1:40 (m/v)) for 2 h at 4°C under stirring. Afterward, the mucilage was removed with a sieve, followed by cheesecloth filtration. The seed trace of the remaining mucilage was removed by contact with ethanol 95% (1:5 (m/v)) for 15 min under the same conditions. Finally, the dry mucilage was extracted from the chia seed surface by mechanical attrition on a filter paper. Mucilage-free seeds were ground into flour using a coffee grinder. Then, the resulting flour was defatted with hexane (1:5 (m/v)) at room temperature, with three solvent changes, and stored at -20°C. This study was registered in the SISGEN: National Management System of Genetic Heritage and Associated Traditional Knowledge under the accession number A879F9E.

Bovine pancreatic trypsin (EC 3.4.21.4), bovine serum albumin (BSA), Na-benzoyl-D,L-arginine4-nitroanilide hydrochloride (BAPNA), sodium dodecyl sulfate (SDS), acrylamide, bis-acrylamide, tricine, dithiothreitol (DTT), 2,7-dichlorofluorescein diacetate (DCFH-DA) and propidium iodide were purchased from Sigma–Aldrich (St. Louis, MO, USA). Standard molecular weight markers (14.4–97.4 kDa) and CNBr-activated Sepharose 4B chromatographic matrix were obtained from GE Healthcare. Trypsin immobilization was carried out according to the manufacturer's instructions. For reversed-phase analysis,

the HPLC Symmetry C18 column (0.46 × 25 cm, 5 µm particle size, 300 Å pore size) was obtained from Waters Corp (Milford, MA, USA). All other chemicals and reagents used were of analytical grade.

## 2.2. *Sh*TI purification

A crude extract (CE) was obtained by using mucilage and fat-free chia seed flour (5 g) in 0.05 M Tris-HCl buffer, pH 8.0, containing 0.5 M NaCl (1:10; m/v) under stirring for 4 h at 4°C. Next, the suspension was filtered through a cheesecloth, the filtrate was centrifuged at 10,000 × g and 4°C for 30 min, and the supernatant was collected. The CE was subjected to heating at 100°C for 30 min. After heat treatment, it was cooled under ice water and centrifuged at 12,000 × g for 15 min at 4°C. The clear supernatant, termed heat-treated crude extract (HCE; 25 mg), was applied to a trypsin-Sepharose 4B column (1.7 × 5.5 cm) equilibrated with the extraction buffer. The adsorbed proteins were eluted with 0.05 M glycine-HCl, pH 2.2, containing 0.5 M NaCl at a 60 mL.h<sup>-1</sup> flow rate (fractions of 3 mL). The retained peak was pooled, dialyzed against Milli-Q grade water, and freeze-dried. The lyophilized sample was dissolved in 0.1% (v/v) trifluoroacetic acid (TFA) in 2% (v/v) acetonitrile and Milli-Q grade H<sub>2</sub>O (eluent A), filtered through a 0.45 µm membrane and then subjected (100 µL) to reversed-phase high-performance liquid chromatography using a C18 column. The proteins were eluted using a linear gradient (0–100% (v/v)) with eluent B (0.1% (v/v) TFA in 80% (v/v) acetonitrile and Milli-Q grade H<sub>2</sub>O) at a 60 mL.h<sup>-1</sup> flow rate. *Sh*TI was collected, freeze-dried, and stored at –20°C for further analysis. Fractions from affinity and reversed-phase chromatography were monitored at 280 nm. Protein concentrations were determined by the dye-binding method described by Bradford [21], with bovine serum albumin (BSA) as the standard. The *Sh*TI purification protocol was guided by a trypsin inhibitory activity assay, which estimated the yield after each purification step.

## 2.3. Trypsin inhibitory activity assay

Trypsin inhibitory activity was assayed as described by Erlanger et al. [22] against bovine trypsin (EC 3.4.21.4) using BApNA as the substrate. Aliquots of trypsin (10 µL; 0.3 mg/mL prepared in 0.001 M HCl) in 690 µL of assay buffer (0.05 M Tris-HCl, pH 8.0, containing 0.02 M CaCl<sub>2</sub>) were incubated with 100 µL of sample test at 37°C for 10 min. CE (0.4 mg/mL), HCE (0.2 mg/mL), retained Trypsin-Sepharose 4B (0.00625 mg/mL) and *Sh*TI (0.00625 mg/mL) were used in triplicate. The reaction was initiated by adding 500 µL of the synthetic chromogenic substrate BApNA (1.25 × 10<sup>-3</sup> M prepared in 1% (v/v) DMSO) and incubating at 37°C for 15 min. The enzyme reaction was stopped by adding 120 µL of 30% (v/v) acetic acid. The release of *p*-nitroanilide from substrate hydrolysis was monitored at 410 nm. One trypsin inhibitory activity unit (TIU) was defined as the decrease in 0.01 U of absorbance per 15 min assay at 37°C, based on enzyme activity in the absence of the inhibitor (control sample).

## 2.4. Biochemical characterization of *Sh*TI

### 2.4.1. Purity and molecular mass determination

The purity from samples of each purification step was checked by tricine-SDS-PAGE as described by Schagger and Von Jagow [23]. Low-range molecular weight standards (14.4–97.4 kDa) were employed

for apparent molecular mass estimation of *ShTI* (GelAnalyzer 2010a software) under nonreducing conditions. Protein bands were revealed by staining the gel with 0.1% (m/v) Coomassie Brilliant Blue R-250 and destaining with distilled water, methanol, and acetic acid (5:4:1– v/v/v).

The average molecular mass of *ShTI* was determined using electrospray ionization-mass spectrometry (ESI-MS). Pure samples of *ShTI* ( $60 \mu\text{M}$ ; prepared in 50% (v/v) acetonitrile containing 0.2% formic acid (v/v)) were applied to a nanoelectrospray source coupled to a Synapt HDMS ESI-Q-ToF mass spectrometer (Waters Corp., Milford, MA, USA) using a Hamilton syringe. The instrument was calibrated with phosphoric acid and operated in positive-ion mode under 3.5 kV capillary voltage at 363 K source temperature. Mass spectra were acquired by scanning at  $m/z$  from 1000 to 2100 and at  $1 \text{ scan}\cdot\text{s}^{-1}$ . Mass Lynx 4.1 software (Waters) was used to deconvolute the mass spectrum.

## 2.4.2. Assessment of the glycoprotein nature of *ShTI*

The detection of the glycoprotein nature of *ShTI* was evidenced by the periodic acid-Schiff (PAS) technique, as reported by Zacharius et al. [24]. Glycoprotein bands were revealed by washing the gel with 0.5% (m/v) potassium metabisulfite in 0.05 M HCl. Fetuin (7  $\mu\text{g}$ ) was used as the positive control.

## 2.4.3. Thermal, pH, and DTT stability of *ShTI*

*ShTI* thermal stability was assessed after incubation at 100°C for 30, 60, 90, and 120 min in a water bath. After heat treatment, the *ShTI* was cooled to room temperature (25 °C). The effect of pH was studied by exposing aliquots of *ShTI* to the following buffers (0.05 M) for 30 min at 25°C: glycine-HCl, pH 2 and 3; sodium phosphate, pH 6; Tris-HCl, pH 8; and glycine-NaOH pH 9 and 10.

The effect of DTT on *ShTI* activity was studied by preincubating samples with the reducing agent (0.1 M) for 30, 60, 90, and 120 min at 37°C. After treatment, iodoacetamide (0.2 M) was immediately added to prevent the formation of disulfide bonds. In all experiments, purified *ShTI* (0.00312 mg/mL) was assayed in triplicate for trypsin inhibitory activity as described in section 2.3. Trypsin inhibitory residual activity was determined (mean  $\pm$  standard deviation) based on each appropriate control not exposed to the respective treatments.

## 2.4.4. Kinetic analysis

The  $\text{IC}_{50}$  value and the inhibition stoichiometry (ratio *ShTI*:trypsin) were estimated through the method described in section 2.3 using purified *ShTI* in the final concentration range of  $1.03 \times 10^{-8} \text{ M}$  to  $2.07 \times 10^{-8} \text{ M}$  [25].

The pattern of enzyme inhibition and the inhibition constant ( $K_i$ ) were analyzed by Lineweaver – Burk double-reciprocal [26] and Dixon [27] plots, respectively. Trypsin inhibitor activity was performed as previously described in section 2.3 by using different concentrations of *ShTI* ( $0.77 \times 10^{-8} \text{ M}$  to  $8.31 \times 10^{-8} \text{ M}$ ) and BApNA ( $0.50 \times 10^{-3} \text{ M}$  to  $1.60 \times 10^{-3} \text{ M}$ ) in the presence of fixed trypsin concentration (0.3 mg/mL). The Lineweaver – Burk plot was drawn by the reciprocal of the reaction rate ( $1/v$ ; OD 410 nm.  $\text{h}^{-1}$

$1 \text{ mL}^{-1}$  of the reaction medium) against the inverse of the substrate concentration ( $1/[S]$ ;  $1/M$ ) in the absence and presence of *ShTI*. The Dixon graph was plotted by the inverse of the reaction rate versus *ShTI* concentration, and  $K_i$  was determined by the intersection of the three lines plotted for different BApNA concentrations ( $0.50$ ,  $1.25$ , and  $1.60 \times 10^{-3} \text{ M}$ ).

## 2.5. Antibacterial activity of *ShTI* and mode of action study

### 2.5.1. Bacterial strains

Antibacterial susceptibility assays were carried out with Gram-negative bacterial strains (*Escherichia coli* ATCC® 8739, *Pseudomonas aeruginosa* ATCC® 9027) and Gram-positive strains (*Staphylococcus aureus* ATCC® 6538P, methicillin-resistant *Staphylococcus aureus* ATCC® 4996, and a clinical strain of MRSA). All strains were obtained from the bacterial collection of the Laboratory of Bioprospecting of Antimicrobial Molecules (LABIMAN), affiliated with the Drug Research and Development Center and the School of Pharmacy at the Federal University of Ceará. First, all strains were grown in Mueller-Hinton agar medium at  $35^\circ\text{C}$  for 24 h. After that, each bacterial inoculum was prepared in a sterile saline solution based on 0.5 McFarland standard. Then, this suspension was diluted in Mueller-Hinton broth to obtain  $1 \times 10^7$  colony forming units (CFU)/mL.

### 2.5.2. MIC determination

The MIC determination was performed by the microdilution broth method as previously published in the M07-A10 document [28]. In brief, the bacterial inoculum was seeded into polystyrene flat-bottom 96-well microtiter plates in the presence of *ShTI* solubilized in distilled water and filtered using  $0.22 \mu\text{m}$  membranes in the concentration range of  $0.03$ – $19.03 \mu\text{M}$ . Sodium oxacillin (Blau Farmacêutica S/A - Cotia, SP, Brazil) was used as the positive control following CLSI guidelines. A control assay in the absence of *ShTI* was also included. Plates were incubated at  $36^\circ\text{C}$  for 20 h. The MIC ( $\mu\text{M}$ ) was determined by identifying the lowest concentration with no visible turbidity. Experiments were carried out in triplicate.

### 2.5.3. Checkerboard assay

Checkerboard titration was employed to calculate the fractional inhibitory concentration index (FICI) and determine the type of interaction between *ShTI* and oxacillin, as reported by Liu et al. [29]. Sub-MIC concentrations of *ShTI* ( $\text{MIC}/32$  –  $\text{MIC}/4$ ) and oxacillin ( $\text{MIC}/32$  –  $\text{MIC}/4$ ) were tested against the three Gram-positive bacterial strains. Sample preparation, inoculum size, and culture conditions were like those described in section 2.5.1. The MIC of each agent alone and in combination was determined as aforementioned (section 2.5.2) and applied in the following formula:  $\text{FICI} = \text{MIC oxacillin combination} / \text{MIC oxacillin isolated} + \text{MIC } ShTI \text{ combination} / \text{MIC } ShTI \text{ isolated}$ . Drug interaction was interpreted as follows:  $\text{FICI} \leq 0.5$ , synergy (SYN);  $0.5 < \text{FICI} < 4.0$ , indifference (IND); and  $\text{FICI} \geq 4.0$ , antagonism (ANT) [30].

### 2.5.4. Cell membrane permeabilization assay

The propidium iodide staining method was employed for the cell membrane permeabilization assay, according to Martins et al. [31]. Methicillin-resistant *Staphylococcus aureus* ATCC® 4996 cells were treated with distilled water (control) or MIC/2 *ShTI* (7.91  $\mu$ M) for 20 h, washed three times with NaCl 0.15 M, followed by centrifugation at low speed (2000 g, 5 min, 25°C). Subsequently, bacteria-treated cells were recovered and incubated with propidium iodide (0.4  $\mu$ L; 0.001 M) for 1 h at 37°C under constant agitation in the dark. Plasma membrane permeabilization was observed with a fluorescence microscope (Olympus BX 41; excitation wavelength, 545 nm; emission wavelength, 580–590 nm; 40X magnification).

### 2.5.5. Reactive oxygen species (ROS) detection

Intracellular ROS generation was assessed by the DCFH-DA staining method described by Martins et al. [31]. MRSA ATCC® 4996 inoculum was treated with distilled water (control) or MIC/2 *ShTI* (7.91  $\mu$ M) and recovered as previously reported in section 2.5.4. Afterward, the cells were incubated with 10  $\mu$ M DCFH-DA at 37°C for 30 min. The presence of ROS was visualized under a fluorescence microscope (Olympus BX 41; excitation wavelength, 490 nm; emission wavelength, 510–530 nm; 40X magnification).

### 2.5.6. Scanning electron microscopy (SEM) analysis

MRSA ATCC® 4996 samples were recovered after treatment with distilled water (control) or MIC/2 *ShTI* (7.91  $\mu$ M) under the conditions described in section 2.5.4. Then, bacterial cell fixation, dehydration, and assembly onto an SEM sample stub were processed according to Martins et al. [31]. SEM analysis was conducted using an Inspect™ 50 FEI scanning electronic microscope (FEI Company, Hillsboro, Oregon, USA).

## 2.6. Statistical analysis

Experimental data were collected in triplicate and expressed as the means  $\pm$  standard deviations. One-way analysis of variance (ANOVA) was used to evaluate significant differences, followed by Tukey's post-hoc test using GraphPad Prism® software (GraphPad Prism, Inc., San Diego, CA, USA). In all comparisons, significant differences were considered at  $p < 0.05$ .

## 3. Results

### 3.1. *ShTI* purification

The trypsin inhibitor from *S. hispanica* L. seeds (*ShTI*) was purified using heat treatment and two chromatographic steps (Trypsin-Sepharose 4B affinity column and Reverse Phase C18 column). First, the crude extract of chia seeds (5 g), containing 606.86 mg of total soluble proteins, was heated at 100°C for 30 min. This procedure rendered a thermostable soluble protein fraction designated heat-treated crude extract (HCE). HCE showed a lower total protein content (206.28 mg) than the crude extract but a higher specific trypsin inhibitory activity, which increased 2.14-fold after heating the crude extract.

As depicted in Fig. 1a, two protein fractions were obtained when HCE was loaded on Trypsin-Sepharose 4B. The non-retained fraction did not show trypsin inhibitory activity and was therefore discarded.

However, the adsorbed fraction eluted with 0.05 M glycine–HCl, pH 2.2, 0.5 M NaCl displayed trypsin inhibitory activity and was recovered and lyophilized.

The RP-HPLC chromatogram of the retained trypsin-Sepharose 4B peak showed a prominent single peak with maximum trypsin inhibitory activity when the acetonitrile gradient reached 57–63% of eluent B at a 44–48 min retention time (Fig. 1b).

The purification levels and yield of each purification step are summarized in Table 1. The purification protocol achieved an 83.29-fold purification with a 16.47% yield based on the total inhibitory activity recovered at the last step of the trypsin inhibitor purification protocol, here named *ShTI*.

Table 1  
*ShTI* purification steps from *Salvia hispanica* seeds

| Purification steps                                                                                                                         | Total protein (mg) <sup>a</sup> | Total inhibitory activity (TIU) <sup>b</sup> | Specific inhibitory activity (TIU.mg <sup>-1</sup> ) | Purification (fold) <sup>c</sup> | Yield (%) <sup>d</sup> |
|--------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------|----------------------------------------------|------------------------------------------------------|----------------------------------|------------------------|
| Crude Extract (CE)                                                                                                                         | 606.86                          | 270,052.70                                   | 445                                                  | 1                                | 100                    |
| Heat-treated crude extract (HCE)                                                                                                           | 206.28                          | 196,997.40                                   | 955                                                  | 2.14                             | 72.94                  |
| Retained Trypsin-Sepharose 4B                                                                                                              | 1.60                            | 56,490.67                                    | 35,306.67                                            | 79.34                            | 20.91                  |
| Reversed-Phase ( <i>ShTI</i> )                                                                                                             | 1.20                            | 44,480                                       | 37,066.67                                            | 83.29                            | 16.47                  |
| a Total protein recovered from defatted chia seed flour (5 g).                                                                             |                                 |                                              |                                                      |                                  |                        |
| b One TIU (trypsin inhibitory activity unit) was defined as the decrease in 0.01 units of absorbance at 410 nm per 15 min assay at 37°C.   |                                 |                                              |                                                      |                                  |                        |
| c Purification index was calculated as the ratio between the specific activity obtained at each purification step and the CE taken as 1.0. |                                 |                                              |                                                      |                                  |                        |
| d Yield was calculated based on the total inhibitory activity recovered (CE, 100%).                                                        |                                 |                                              |                                                      |                                  |                        |

Tricine-SDS–PAGE analysis of *ShTI* revealed a single protein band with an apparent molecular mass of 11 kDa under nonreducing conditions (Fig. 1b - Insert). Furthermore, *ShTI* was stained with Schiff reagent dyes, indicating a carbohydrate moiety covalently bound in its structure, confirming that the purified inhibitor is a glycoprotein (Fig. 1b - Insert). Regarding native molecular mass, *ShTI* exhibited a major ion of 11,558 Da in the deconvoluted ESI-MS spectrum (data not shown).

### 3.2. *ShTI* stability

*ShTI* proved to be highly thermostable, maintaining 100% of its trypsin inhibitory activity even when incubated at 100°C for up to 2 h (Fig. 2a). Moreover, the purified trypsin inhibitor was stable over a broad pH range (2–10). *ShTI* retained 80–100% of its inhibitory activity at acidic and basic pH values (Fig. 2b).

In contrast, *Sh*TI trypsin inhibitory activity was affected by DTT (reducing agent). The inhibitory activity of *Sh*TI significantly declined to ~ 77% after 30 min of treatment with DTT (0.1 M). A longer exposure time to DTT reduced its activity to 62% (Fig. 2c).

### 3.3. IC<sub>50</sub> and kinetic study

According to the linear regression shown in Fig. 3a (equation:  $y = 28.64x$ ;  $R^2: 0.99$ ), the IC<sub>50</sub> of *Sh*TI toward trypsin was  $1.74 \times 10^{-8}$  M. Based on the titration curve (Fig. 3b), *Sh*TI inhibits trypsin in a molar ratio of 1:1. Lineweaver-Burk and Dixon plots were analyzed to determine the inhibition mechanism by which *Sh*TI inhibits trypsin and the dissociation constant ( $K_i$ ). In the double-reciprocal plot (Fig. 4a), increasing concentrations of *Sh*TI led to an increase in  $K_m$  with no effect on  $V_{max}$ , indicating a competitive mode of inhibition. In the Dixon plot (Fig. 4b), intersecting lines that converge above the x-axis were used to calculate  $K_i$ . The  $K_i$  value obtained for *Sh*TI was  $1.79 \times 10^{-8}$  M.

### 3.4. Antibacterial activity of *Sh*TI

*Sh*TI exhibited antibacterial activity against all three *S. aureus* strains, showing MICs ranging from 15.83 to 19.03  $\mu$ M (Table 2), the lowest MIC value found against MRSA ATCC® 4996. In contrast, no significant effect was observed toward the Gram-negative bacteria tested. Moreover, *Sh*TI also presented a synergic action when combined with oxacillin, as depicted in Table 3. FICI values ranging from 0.20 to 0.33 were found. The clinical *S. aureus* strain was not susceptible to cotreatment with *Sh*TI and oxacillin.

| Table 2<br>Minimum inhibitory concentrations (MICs) of <i>Sh</i> TI against the standard and clinical bacterial strains                                                                   |                             |
|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------|
| Bacterial strains                                                                                                                                                                         | MIC ( $\mu$ M) <sup>b</sup> |
| <i>S. aureus</i> ATCC® 6538P                                                                                                                                                              | 19.03                       |
| MRSA ATCC® 4996                                                                                                                                                                           | 15.83                       |
| Clinical MRSA <sup>a</sup>                                                                                                                                                                | 19.03                       |
| <i>E. coli</i> ATCC® 8739                                                                                                                                                                 | > 19.03                     |
| <i>P. aeruginosa</i> ATCC® 9027                                                                                                                                                           | > 19.03                     |
| a Bacterial strain isolated from a biological sample.                                                                                                                                     |                             |
| b The MIC (minimum inhibitory concentration) value was defined as the lowest concentration that produced no visible growth of bacterial cells in the plate well after 20 h of incubation. |                             |

Table 3  
Synergistic activity between *Sh*TI and oxacillin against *S. aureus*

| MIC (μM)                                                                                                          |          |           |          |           |                   |                             |
|-------------------------------------------------------------------------------------------------------------------|----------|-----------|----------|-----------|-------------------|-----------------------------|
|                                                                                                                   | Isolated |           | Combined |           | FICI <sup>a</sup> | Interpretation <sup>b</sup> |
| Bacterial strains                                                                                                 | ShTI     | Oxacillin | ShTI     | Oxacillin |                   |                             |
| <i>S. aureus</i> ATCC® 6538P                                                                                      | 19.03    | 1.24      | 3.37     | 0.19      | 0.33              | SYN                         |
| MRSA ATCC® 4996                                                                                                   | 15.83    | 39.85     | 1.66     | 4.18      | 0.20              | SYN                         |
| Clinical MRSA                                                                                                     | 19.03    | 159.43    | > 10.38  | 79.71     | 1.04              | IND                         |
| a: The fractional inhibitory concentration index (FICI) was obtained using the following formula:                 |          |           |          |           |                   |                             |
| FICI = MIC oxacillin combination/MIC oxacillin isolated + MIC <i>Sh</i> TI combination/MIC <i>Sh</i> TI isolated. |          |           |          |           |                   |                             |
| b: Interpretation was carried out based on FICI values as follows:                                                |          |           |          |           |                   |                             |
| FICI ≤ 0.5: synergism (SYN); 0.5 < FICI ≤ 4.0: indifferent (IND); FICI > 4.0: antagonistic (ANT).                 |          |           |          |           |                   |                             |

Once MRSA ATCC® 4996 was the most sensitive strain to *Sh*TI, it was selected to assess the antibacterial mode of action of *Sh*TI. Fluorescence microscopy analysis was employed to observe membrane permeabilization and the induction of ROS generation (Fig. 5). No fluorescence signal was observed for the control group (Fig. 5e and g). In contrast, *Sh*TI-treated cells (MIC/2: 7.91 μM) exhibited red fluorescence caused by the presence of propidium iodide, indicating cell membrane damage (Fig. 5f). Additionally, *Sh*TI triggered ROS formation, as evidenced by bright green fluorescence detection (Fig. 5h).

Scanning electron microscopy (SEM) was used to observe the effects of *Sh*TI treatment in *S. aureus* cells (Fig. 6). Untreated MRSA ATCC® 4996 cells did not exhibit cell morphology alteration, presenting a smooth surface with an intact plasma membrane (Fig. 6a). However, after exposure to MIC/2 *Sh*TI (7.91 μM), *S. aureus* cells displayed surface ruptures with remarkable membrane pore formation, as observed for three different zones imaged (Fig. 6b – d).

## 4. Discussion

*Salvia hispanica* L. seeds are a well-consumed food in different countries worldwide due to their numerous benefits to human health [32]. Its seeds are rich in nutraceutical compounds, resulting in high nutritional and health/medical values [13, 32]. Today, their incorporation - whole or flour - into yogurt, salads, bread, cakes, and others is the main form of consumption. Overall, chia seeds provide a new opportunity for their consumption as a functional food and a source of safe bioactive compounds with benefits to human health [13]. Indeed, the in vitro biological activities of its digested protein fractions have been reported [16–19]. However, studies with isolated proteins from chia seeds are still scarce, despite their biotechnological applications. Among these compounds, protease inhibitors represent a

large group of molecules in plant seeds with high agronomical, biotechnological, and biomedical added value [10–12], which has already been found in chia seeds but is still unexplored [20].

Many biological activities have been reported for PPIs, such as antitumoral, antioxidant, anticoagulant, and insecticidal [12]. They have also been tested against osteoporosis, cardiovascular and inflammatory diseases, neurological disorders, and antimicrobial agents [33]. Therefore, PPIs offer a remarkable opportunity for drug discovery and development. In this sense, considering their diversity already identified (> 6700 plant-derived proteinaceous PIs) and those still unexplored, determining the potential effects of each novel PPI on human health is of great interest [11, 33].

The initial *Sh*TI purification steps involved heat treatment and affinity chromatography. These combined methods have been successfully employed for PPI purification [31, 34, 35]. In fact, due to the high physicochemical stability commonly associated with PPIs, heat treatment is carried out at the initial purification steps with minimal loss of inhibitory activity. Such a procedure allows the elimination of thermolabile proteins while concentrating the thermostable protein of interest [31, 34, 35]. Affinity chromatography was also an efficient and convenient step for *Sh*TI purification that allowed us to remove the most contaminants present in HCE, as observed in the tricine-SDS–PAGE profile. Nevertheless, a reversed-phase C18 column was used in the purification process as a final step to obtain *Sh*TI, showing a homogeneous single protein band after tricine-SDS–PAGE.

Previously, Souza et al. [20] isolated a trypsin inhibitor from commercial chia seed and flour in a protocol involving protein extraction, ammonium sulfate fractionation, and affinity chromatography. This protocol led to a low yield, and the soluble protein content of the retained peak from trypsin Sepharose CNBr 4B was detected in a discrete amount (0.001 mg/mL) by the Bradford method. Because of this, it was not possible to determine the specific activity. In contrast, the purification process applied in this work proved to be more efficient, achieving a 0.19% *Sh*TI protein yield based on the protein content present in the crude extract. This value is similar to those found in *Rc*TI from *Ricinus communis* L. cake (0.1%) [34] and *Lza*BI, a Bowman-Birk protease inhibitor from *Luetzelburgia auriculata* seeds (0.2%) [31].

After purification of *Sh*TI, its physicochemical characterization was performed. *Sh*TI showed an apparent molecular mass of approximately 11 kDa, as evidenced by tricine-SDS–PAGE under nonreducing conditions and confirmed by mass spectrometry analysis (11.558 kDa). This molecular mass is close to those reported for other trypsin inhibitors, such as *Jc*TI from *Jatropha curcas* seed cake (10.252 kDa) [25] and *Rc*TI (14 kDa) [34]. Although *Sh*TI was characterized as a glycoprotein, the presence of carbohydrate moieties covalently bound to trypsin inhibitor structures is not a consensus. While *Jc*TI [25] and *Av*TI from *Acacia victoriae* seeds [36] were also classified as glycosylated proteins, *Rc*TI [34] and *C*TI from *Cassia leiandra* seeds [37] did not show covalently linked carbohydrates.

PPIs are remarkably stable to temperature, pH, chemical agents (detergents, reductants, and oxidants), ionic strength, and proteolysis. Such characteristics are attributed to their compact tridimensional structure linked by disulfide bonds. Noncovalent interactions (e.g., hydrogen bonds and electrostatic interactions) also contribute to protein folding and overall stability [12, 38]. Considering the importance of

PPI physical and chemical stability for biotechnological applications, *ShTI* stability was studied under prolonged exposure to extreme temperatures (100°C; 120 min), a wide range of pH values, and in the presence of a reducing agent (DTT).

*ShTI* proved to be thermostable and resistant to a wide range of pH values. Due to the rigidity of the trypsin inhibitor scaffold, in the face of high-temperature treatment and an extensive range of pH values, small changes in the tridimensional structure can be observed [12, 38]. Molecules with physicochemical stability are of great interest to agricultural, industrial, and pharmaceutical applications. These compounds do not demand special storage conditions (e.g., cold or warm temperatures), formulations with stabilizers, preservation in buffers, and a nonoxidative environment. Furthermore, they can resist the adverse conditions of the gastrointestinal tract, which is essential for the development of orally administered drugs [12]. When exposed to 0.1 M DTT treatments (30–120 min.), *ShTI* trypsin inhibitory activity was partially reduced (23–38% loss), reinforcing the involvement and importance of disulfide bridges in its native structure.

PPIs can act reversibly or irreversibly regarding the inhibition mechanism to target proteases [11]. Stoichiometric studies with *ShTI* and trypsin indicated an inhibitor:enzyme molar ratio of 1:1, showing an  $IC_{50}$  of  $1.74 \times 10^{-8}$  M. The result suggests that *ShTI* presents a single reactive site trypsin complex formation, as also observed for *IvTI* (*Inga vera* trypsin inhibitor) [39] and *EaTI* (*Entada acaciifolia* trypsin inhibitor) [40]. Kinetic studies of trypsin by *ShTI* described a competitive inhibition mode of action with a low  $K_i$  value of  $1.79 \times 10^{-8}$  M, indicating high affinity toward trypsin. Different degrees of interaction have been determined for the trypsin and PPI relationship. For instance, a similar affinity toward trypsin was described for *RcTI* ( $1.90 \times 10^{-8}$  M) [34], while *EaTI* ( $1.75 \times 10^{-9}$  M) showed a more potent degree of interaction [40]. Notably, the low  $K_i$  value found in PPIs has prompted their use in preclinical studies, highlighting their potential as therapeutic agents in drug development [33]. In this sense, *ShTI* may also be a promising candidate to be exploited.

In the biomedical field, extensive studies have revealed the antibacterial activity of PPIs alone [25, 31, 39, 41]. However, strategies involving their effects in drug combination therapies have not been well explored. Therefore, we investigated the antibacterial properties of *ShTI* independently and in simultaneous treatment with oxacillin, a conventional antibiotic in clinical practice.

This study found that *ShTI* showed selective activity against *S. aureus*, including MRSA strains (MICs range from 15.83 to 19.03  $\mu$ M). In contrast, no significant inhibitory effect was observed against Gram-negative bacteria. Generally, the antibacterial properties of PPIs are attributed to their ability to inhibit the activity of proteases from microorganisms, resulting in antinutritional outcomes [8, 25, 41]. In addition, perturbations of the membrane permeability of pathogens have also been reported [8, 31, 41].

*Staphylococcus aureus* represents an important Gram-positive human pathogen involved in most nosocomial and community-acquired infections worldwide. In the past,  $\beta$ -lactam antibiotics have been the first line for *S. aureus* infection treatment, including oxacillin and second-generation penicillin.

However, the emergence of MRSA made early-generation  $\beta$ -lactam antibiotics no longer be used clinically [4, 42]. In this context, the World Health Organization (WHO) has issued a global priority pathogens list, including MRSA as a high priority group for research and development of new therapeutics [43]. Here, *ShTI* interacted synergistically with oxacillin against sensitive and MRSA strains. Barros et al. [44] also observed a positive interaction between antibiotics (ciprofloxacin and vancomycin) and EpTI (a trypsin inhibitor from *Erythrina poeppigiana* seeds) against *Klebsiella pneumoniae* and *S. aureus*. Several advantages have been attributed to combination therapies over monotherapy, such as broadening the microorganism spectrum susceptible to available drugs, decreasing the risk of resistance development, diminishing therapeutic doses and treatment durations, and minimizing adverse effects [42].

To evaluate the antibacterial action mode of *ShTI*, MRSA-treated cells were analyzed under fluorescence and scanning electron microscopy. Its ability to disrupt cell membranes was demonstrated as indicated by intracellular propidium iodide detection. Propidium iodide has a strong affinity to interact with DNA in a complex that emits red fluorescence. However, as a membrane-impermeable fluorescent dye, it can only internalize cells with compromised plasma membranes. Disbalance into membrane permeabilization can lead to cytoplasmic content loss, resulting in cell death [31]. The *ShTI* pore-forming capacity was confirmed by SEM.

Martins et al. [31] estimated a protein size of 3.62 nm in diameter of *LzaBBI* (17.3 kDa) by using a theoretical tool ([http://www.calctool.org/CALC/prof/bio/protein\\_size](http://www.calctool.org/CALC/prof/bio/protein_size)). At the same platform, *ShTI* showed a theoretical diameter of 3.15 nm. Thus, considering the porous structure of Gram-positive bacterial cell wall dimensions of 40–80 nm, it is reasonable to assume that *ShTI* can reach and interact with the *S. aureus* plasma membrane, disturbing its surface, as suggested by Martins et al. [31]. Notably, the cell wall of Gram-negative bacteria possesses an outer membrane that prevents the passage of some antibacterial compounds that are not present in Gram-positive microorganisms [1]. This intrinsic resistance attributed to the cell wall structure might explain the particular activity found to *ShTI* against those pathogens.

*ShTI* also induced the production of reactive oxygen species (ROS) in *S. aureus*. Overproduction of ROS can lead to a toxic environment for microorganisms, in which damage to proteins, DNA, and lipids may occur, finally resulting in cell death. Although other PPIs have also been involved in inducing ROS accumulation in bacterial and fungal cells [9, 31], their mechanisms are not well understood. It has been suggested that ROS overproduction results from the activation of mitochondria to assure the increased energetic metabolism necessary to repair and maintain membrane cell physiological function, which was previously damaged [9].

Taken together, considering the mode of action of oxacillin and *ShTI*, it is reasonable to assume that oxacillin may perturb cell wall structure, which in turn improves the entry and interactions between *ShTI* and the plasma membrane, thereby generating the observed synergistic effects. Thus, considering the potential therapeutic application of *ShTI* in treating infections caused by *S. aureus* alone or in drug combination therapy, its toxicological assessment and in vivo responses deserve further study.

## 5. Conclusion

Herein, a trypsin inhibitor from *S. hispanica* L. seeds was purified and characterized. *ShTI* shares remarkable stability to high temperature and a broad pH range usually found in PPIs, which seems to be related with the presence of disulfide bridges. *ShTI* displayed antibacterial activity toward sensitive and resistant strains of *S. aureus* via oxidative stress by ROS overproduction and membrane permeabilization disbalance by membrane cell pore formation. Furthermore, *ShTI* improved the therapeutic potency of oxacillin against *S. aureus*, including an MRSA strain. Thus, *ShTI* represents a novel candidate for use as a therapeutic agent for the bacterial management of *S. aureus* infections.

## Declarations

### Credit authorship contribution statement

**Adson Ávila de Souza:** conceptualization, methodology design, validation, investigation, data curation, formal analysis, writing - original draft and writing - review & editing. **Adrienne Maia Lima:** investigation. **Daniele de Oliveira Bezerra de Sousa:** funding acquisition, writing - initial draft and writing - review & editing. **Francisca Cristiane Nogueira:** investigation. **José Carlos do Sacramento Neto:** investigation. **Lucas Pinheiro Dias:** research and formal analysis. **Nadine Monteiro Salgueiro Araújo:** investigation and formal analysis. **Celso Shiniti Nagano:** investigation, formal analysis, funding acquisition, writing - original draft and writing - review & editing. **Hélio Vitoriano Nobre Júnior:** funding acquisition, writing - initial draft and writing - review & editing. **Cecília Rocha da Silva:** funding acquisition, writing - original draft and writing - review & editing. **Lívia Gurgel do Amaral Valente Sá:** investigation and formal analysis. **João Batista de Andrade Neto:** writing - initial draft and writing - review & editing. **Fátima Daiana Dias Barroso:** investigation and formal analysis. **Maria Elisabete Amaral de Moraes:** funding acquisition, writing - original draft and writing - review & editing. **Hermógenes David de Oliveira:** conceptualization, formal analysis, data curation, supervision, funding acquisition, writing - initial draft and writing - review & editing.

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### Declaration of competing interest

The authors declare no conflicts of interest.

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## Figures

**Fig. 1**

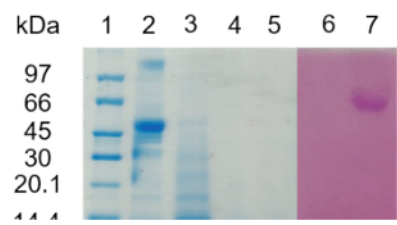


**Figure 1**

Chromatogram profiles and polyacrylamide gel electrophoresis of *ShTI* purification. (a) Heat-treated extract from *S. hispanica* seeds was applied to a Trypsin-Sepharose column. (b) Reversed-phase chromatography of the retained trypsin-Sepharose fraction eluted with a linear gradient from 100% eluent A (0.1% (v/v) TFA in 2% (v/v) acetonitrile) to 100% eluent B (0.1% (v/v) TFA in 80% (v/v) acetonitrile). Insert: Tricine-SDS-PAGE (16.5%) of *ShTI*: (1) molar mass markers (MM - kDa); (2) crude extract (CE); (3) heat-treated crude extract (HCE); (4) retained trypsin-Sepharose 4B; (5) purified *Salvia hispanica* trypsin inhibitor (*ShTI*); (6) and (7) *ShTI* and fetuin (positive control) stained with Schiff's reagent for carbohydrate detection, respectively

**Figure 2**

Stability analysis of *ShTI*. (a) *ShTI* was exposed for different incubation periods (30 – 120 min) at 100 °C. (b) *ShTI* was incubated at different pH values for 30 min. (c) *ShTI* was set with DTT (0.1 M) for various incubation periods (30 – 120 min.) at 37 °C. The residual trypsin inhibition activity of *ShTI* was determined based on each appropriate control. The values represent the means of three independent experiments (mean ± standard deviation). Different letters indicate significant differences among the groups (p < 0.05)

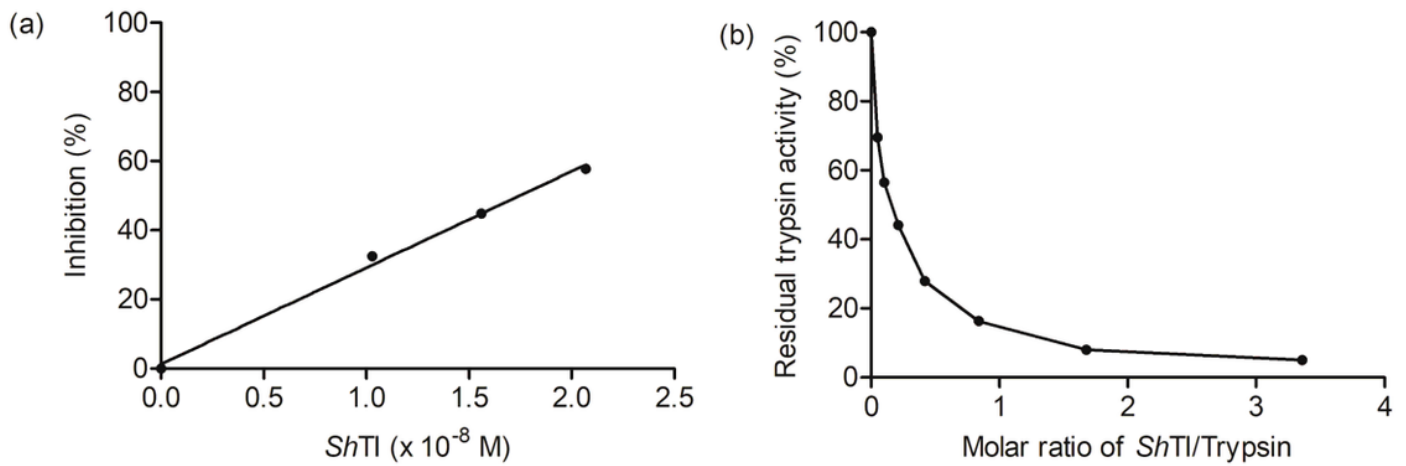


**Figure 3**

Inhibitory activity of *ShTI* against bovine trypsin. (a) A trypsin inhibition curve was built by increasing amounts of *ShTI* at a fixed BA<sub>p</sub>NA concentration ( $1.25 \times 10^{-3}$  M). The  $IC_{50}$  of *ShTI* on trypsin was  $1.74 \times 10^{-8}$  M. (b). The titration curve reveals a 1:1 stoichiometric ratio of *ShTI*-trypsin complex formation

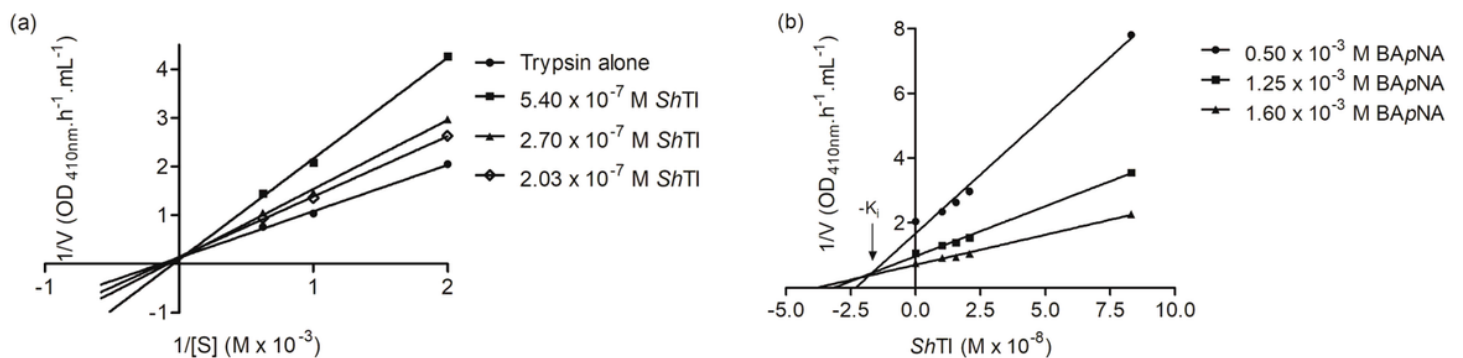


**Figure 4**

Inhibition kinetics of *ShTI* toward bovine trypsin. (a) Lineweaver–Burk plot describes a competitive mechanism of inhibition by *ShTI* at various concentrations of BA<sub>p</sub>NA (0.50, 0.80, 1.00, 1.25, and 1.60  $\times 10^{-3}$  M). (b) A Dixon plot was employed to assess the dissociation constant ( $K_i$ ) of *ShTI* ( $1.79 \times 10^{-8}$  M)

**Figure 5**

Fluorescence microscopy of *S. aureus* cells after distilled water (control) or MIC/2 *ShTI* incubation. Propidium iodide uptake evidenced by red fluorescence indicates cell membrane permeabilization. Reactive oxygen species (ROS) generation was observed in MIC/2 *ShTI* ( $7.91 \mu M$ )-treated cells, as

indicated by high green fluorescence. a, b, c, and d images were recorded in the light field, while e, f, g and h represent fluorescence micrographs. Bars = 20  $\mu\text{m}$

## Figure 6

Scanning electron microscopy (SEM) analysis of *S. aureus* cells exposed to distilled water (a, control) or MIC/2 *ShTI* (b, c and d). The untreated cells showed an intact cell morphology with well-defined and organized structures. *S. aureus*-treated cells (MIC/2 *ShTI*, 7.91  $\mu\text{M}$ ) exhibited deleterious structural changes, as evidenced by of pores on the surface. Bars = 2  $\mu\text{m}$