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A new Kunitz trypsin inhibitor from Erythrina poeppigiana exhibits antimicrobial and antibiofilm properties against bacteria

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ABSTRACT

Erythrina poeppigiana belongs to Fabaceae family (subfamily *Papillionoideae*) and is commonly found in tropical and subtropical regions in Brazil. Herein, we described the purification and characterization of a new Kunitz-type inhibitor, obtained from *E. poeppigiana* seeds (EpTI). EpTI is composed by three isoforms of identical amino-terminal sequences with a molecular weight ranging from 17 to 20 kDa. The physicochemical features showed by EpTI are common to Kunitz inhibitors, including the dissociation constant (13.1 nM), stability against thermal (37–100 °C) and pH (2–10) ranging, and the presence of disulfide bonds stabilizing its reactive site. Furthermore, we investigated the antimicrobial, anti-adhesion, and anti-biofilm properties of EpTI against Gram-positive and negative bacteria. The inhibitor showed antimicrobial activity with a minimum inhibitory concentration (MIC, 5–10 μM) and minimum bactericidal concentration (MBC) of 10 μM for *Enterobacter aerogenes*, *Enterobacter cloacae*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, and *Staphylococcus haemolyticus*. The combination of EpTI with ciprofloxacin showed a marked synergistic effect, reducing the antibiotic concentration by 150%. The increase in crystal violet uptake for *S. aureus* and *K. pneumoniae* strains was approximately 30% and 50%, respectively, suggesting that the bacteria plasma membrane is targeted by EpTI. Treatment with EpTI at 1x and 10 x MIC significantly reduced the biofilm formation and prompted the disruption of a mature biofilm. At MIC/2, EpTI decreased the bacterial adhesion to polystyrene surface within 2 h. Finally, EpTI showed low toxicity in animal model *Galleria mellonella*. Given its antimicrobial and anti-biofilm properties, the EpTI sequence might be used to design novel drug prototypes.

1. Introduction

According to the World Health Organization (WHO), bacterial resistance to antibiotics will be the leading cause of death worldwide in the next decades. Up to 2050, the number of deaths related to bacterial infection and sepsis can reach ten million, overcoming other important diseases such as cancer [1]. Thus, the development of novel therapeutics to treat multidrug-resistant bacteria is imperative. The "ESKAPE" pathogens are a group of bacteria that have garnered attention for their ability to escape or evade common therapies through antimicrobial resistance. In 2008, ESKAPE pathogens were first defined and consist of *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp. [2]. Alternative therapies, such as the use of antibiotics in combination or

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with adjuvants, bacteriophages, antimicrobial peptides, nanoparticles, and photodynamic light therapy are widely reported [3].

Although, a further concern related to microorganism infections lies in the ability of some strains to form a bacterial biofilm. Biofilms comprise a consortium of microorganisms that are protected by an extracellular matrix composed of polysaccharides, proteins, lipids, and nucleic acids [4]. In addition to human body surfaces, microorganisms can adhere and form biofilms on abiotic surfaces, such as medical devices [5,6]. The biofilm's microenvironment facilitates the exchanges of nutrients and shelters from harmful factors, such as the host body's immune system and antibiotics. For this reason, to control biofilms with conventional antibiotics is so hard to be reached. Since bacteria can transmit and acquire resistance to drugs that are currently used as therapeutic agents [7], finding molecules to treat bacterial infections with different mechanisms of action is necessary. In this important search for new effective antimicrobial compounds, several molecules have been tested, including plant peptidase inhibitors (PIs), molecules which have been investigated due their multiple roles in plant defense physiology [8].

PIs are found constitutively in several plant tissues and their expression might be modulated by different conditions, such as herbivory, microorganisms attack, injuries, or environmental stress conditions. Beyond their role in plant defense, PIs have been investigated for their antifungal and antibacterial properties [9]. Several families of PIs are found in plants. Among them, the Kunitz inhibitor is one of the most widely investigated. Currently, Kunitz inhibitors are grouped in family I3 in MEROPS Database [10] and contain PIs of serine and other peptidases. Kunitz inhibitors are formed by proteins of 16–24 kDa, constituted by single or double polypeptide chains with a variable number of disulfide bonds [8].

Regards its biological properties, Kunitz inhibitors show insecticide, antimicrobial and anticancer properties. Some of these activities have been reported by PIs isolated from Brazilian flora. The trypsin inhibitor from *Inga vera* seeds (IVTI) exhibits insecticidal, antimicrobial, and anticancer activities, demonstrating the multifaceted potential of PIs as biotechnological tools for agriculture and healthcare [11,12]. Another PI from *the Inga* genus was purified from *Inga laurina*, named ILTI (*I. laurina* trypsin inhibitor) [11]. ILTI displayed insecticide activity against important insect pests, such as *Spodoptera frugiperda* [13]. Recently, the recombinant ILTI, produced in *Komagataella phaffii* showed antibiofilm and anticancer properties [14]. For these reasons, PIs might offer templates for the rational design of new drugs [15].

Our group researches the biodiversity of the Cerrado, a savanna-like biome, located in the Brazilian Midwest. Cerrado covers about 2 million km^2 of the national territory, where there are many unexplored flora. In this context, we describe the purification and characterization of a new Kunitz trypsin inhibitor, isolated from *Erythrina poeppigiana* seeds. *E. poeppigiana* belongs to the Fabaceae family (subfamily *Papillionoideae*) and is commonly found in tropical and subtropical regions [16]. Once purified, we carried out the physicochemical characterization of EpTI and investigated its antimicrobial and anti-biofilm proprieties.

2. Material and methods

2.1. Plant material

E. poeppigiana seeds were purchased from ArboCenter company seed trade, (lot: 00316, 2012 crop) and were stored (− 20 °C) in the laboratory for protein purification and its biological functions seed bank, Federal University of Mato Grosso do Sul, Campo Grande, Mato Grosso do Sul, Brazil.

2.2. Purification of EpTI

Seeds teguments were manually removed, cotyledons were ground, defatted with hexane, and used in a classical protein extraction methodology [11]. A crude extract (CE), composed by all soluble proteins extracted from seed flour, was obtained by extraction with 100 mM sodium phosphate buffer, pH 7.6 (1:10; w/v) overnight at 4 °C. The solution was centrifugated at 12,500 x *g* for 30 min at 4 °C. The supernatant fraction was dialyzed (dialysis membrane with 12 kDa cut-off) for 48 h against distilled water, lyophilized, and denominated crude extract.

For EpTI purification, the 200 mg of CE were dissolved in 2 ml of 50 mM Tris-HCl buffer, pH 8.0, and loaded onto a Diethylaminoethyl (DEAE)-Sepharose column (3 × 12 cm, Cytiva Life Sciences) equilibrated with the same buffer. Proteins adsorbed to the column were eluted in a 0–1 M NaCl gradient at a flow rate of 3 ml min^{-1}. The 35 fractions collected were assayed looking for inhibitory activity against trypsin. The fractions with confirmed inhibitory activity were pooled, dialyzed, lyophilized, and loaded (10 mg) onto a bioaffinity column trypsin-Sepharose (2 × 9.1 cm, Cytiva Life Sciences), equilibrated with 100 mM sodium phosphate buffer, pH 7.6 containing 100 mM NaCl. Following the washing step, the proteins were eluted with 50 mM HCl at a flow of 1 ml min^{-1}. The collected peak was assayed against trypsin, dialyzed, and lyophilized. All purification steps were monitored by chromatograms, elaborated from the analysis of the protein content of fractions, determined at 280 nm. The protein quantification was determined using the Bradford reagent, using bovine serum albumin (BSA) (Sigma-Aldrich, Sao Paulo, Brazil) as standard [17].

2.3. Inhibitory activity against trypsin

The inhibitory activity against trypsin was determined according to Oliveira et al. [18] using the colorimetric substrate N-benzoyl-DL-arginine-p-nitroanilide (BAPNA; Sigma–Aldrich). An aliquot (4 μL) of bovine trypsin at 0.25 mg ml^{-1} (Sigma–Aldrich) was mixed with 50 mM Tris–HCl, pH 8.0, and EpTI (total volume of 66 μL) for 15 min. Then, 200 μL of 1 mM BAPNA was added to assay, with a total time of 30 min. The kinetic assays were carried out at 30 °C and absorbance at 410 nm was used to monitor the hydrolysis of BAPNA in a Multiskan GO (Thermo Scientific). One unit of the enzyme (U) was defined as the amount of enzyme able to increase the absorbance 0.01 at 410 nm, resulted by substrate hydrolysis.

2.4. Polyacrylamide gel electrophoresis

The purification process was accompanied by 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), according to Laemmli [19], using molecular weight standards (14.4–97.4 kDa, Cytiva Life Sciences). The gels were stained with 0.1% (w/v) Coomassie Brilliant Blue R-250 and distained with a solution of distilled water, methanol, and acetic acid (5:4:1 – v/v/v).

2.5. Detection of isoforms by negative staining

Samples from CE, and the peaks from DEAE-Sepharose and trypsin-Sepharose columns were submitted to SDS-PAGE, to accompany the purity and molecular homogeneity. Since the sample from the trypsin-Sepharose column, supposedly EpTI, showed three bands in SDS-PAGE, we further investigated the occurrence of isoforms using the negative staining with aminophenyl-ethyl and tetrazolium chloride APNE/TETRA [20]. An SDS-PAGE was carried out, following by the fixation with trichloroacetic acid (12.5%) for 30 min. The gel was incubated in 100 mM phosphate buffer pH 7.4 for 30 min and the buffer was replaced by a solution of trypsin (0.1 mg ml^{-1}) prepared in 100 mM phosphate buffer pH 7.4, following a new incubation by 30 min at 30 °C. The gel was stained with APNE/TETRA solution in a ratio of 1:9, respectively. Clear bands revealed against a pink background represented the presence of trypsin inhibitors.

2.6. Amino-terminal sequencing

The proteins from SDS-PAGE were transferred for a polyvinylidene fluoride (PVDF) membrane. The transfer was performed at 300 mA for 45 min using Towbin buffer (25 mM Tris, 192 mM glycine, and 20% methanol). The three bands observed for EpTI from trypsin-Sepharose were cut and assembled into a reactor of a Shimadzu Co PPSQ-33B (Kyoto, Japan) automated protein sequencer, performing Edman degradation [11]. The equipment was previously calibrated with PTH-amino acids mixture standard. The phenylthiohydantoin-amino acid derivatives were detected after separation on an RP-HPLC C18 column (4.6 × 250 mm) under isocratic conditions. The amino-terminal sequence of EpTI bands were determined and the sequences were used to determine the percentage of identity with trypsin inhibitors through the NCBI protein BLAST search system. Multiple alignments were performed using the T-COFFEE Server [21].

2.7. Stability of inhibitory activity against bovine trypsin

The stability of EpTI against trypsin was evaluated in different conditions [11]. A solution of 25 μM EpTI (in 50 mM Tris-HCl pH 8.0) was heated for 30 min at different temperatures (37–100 °C) and the residual inhibitory activity was assayed against trypsin. For pH stability, EpTI was incubated with 100 mM sodium citrate (pH 2–4), 100 mM sodium acetate (pH 5), 100 mM sodium phosphate (pH 6–7), 100 mM Tris-HCl (pH 8) and 100 mM sodium bicarbonate (pH 9–10). After incubation for 1 h at 30 °C, the residual inhibitory activity was assayed as previously described. To assess the presence of disulfide bonds in EpTI, the inhibitor (25 μM) was incubated with DTT at different concentrations (1, 10, and 100 mM) for 15, 30, 60, and 120 min at 30 °C. The reaction was terminated by the addition of iodoacetamide at twice the amount of each DTT concentration, and the inhibitory activity was determined. All experiments were performed in triplicates and the results were expressed as mean ± standard deviation.

2.8. Determination of dissociation constant

A curve with increasing concentrations of EpTI (0; 0.10; 0.25; 0.50; 0.75 and 1 nM) incubated for 10 min at 30 °C with trypsin at a concentration of 5.5 nM. Substrate hydrolysis was monitored at 410 nm. To determine the dissociation constant, different concentrations of BAPNA (1; 0.75 and 0.5 mM) were used with a different inhibitor concentration (0; 0.10; 0.25; 0.50; 0.75 and 1.0 nM). The inverse of the rate (1/v) *versus* inhibitor concentration [I] for each substrate concentration, [S1] and [S2] was plotted with aid of Sigma Plot (Systat Software Inc.). The regression line for each [S] was obtained and the inhibition constant (Ki) was calculated from the intersection of the lines.

2.9. Determination of MIC and MBC

The minimum inhibitory concentration (MIC) of EpTI was determined against *E. coli* (ATCC 35218), *Enterobacter aerogenes* (ATCC 13048), *Enterobacter cloacae* (ATCC 13047), *Klebsiella pneumoniae* (ATCC 70603), *Serratia marcescens* (ATCC 13880), *Acinetobacter baumannii* (ATCC 19606), *Salmonella enterica* (ATCC 51741), *Proteus vulgaris* (ATCC 8424), *Pseudomonas aeruginosa* (ATCC 27853), *Staphylococcus aureus* (ATCC 25923), *Staphylococcus haemolyticus* (ATCC 29970) and *Staphylococcus saprophyticus* (ATCC 29453). Antimicrobial activity was determined by means of minimal inhibitory concentration (MIC) assays, determined by the microdilution technique according to Clinical and Laboratory Standards Institute (CLSI) protocols M07-A9 [22]. The assays were performed in 96-well microplates. The culture medium used was Muller Hinton (MH) for bacterial strains. Inoculums were standardized by reading a spectrophotometer at 700 nm for bacteria. MIC was defined as the lowest concentration of compounds that inhibited visible microbial growth. With these results, we selected the best bacterial strain to proceed with the tests. EpTI were diluted in PBS and assayed from 400 μg ml^{-1} (20 μM) to 3.9 μg ml^{-1} (1.95 μM). Ciprofloxacin and vancomycin were used as a positive control for bacteria growth. The minimum bactericidal concentration (MBC) was determined by subculturing aliquots of 10 μL of the concentrations onto BHI (Difco) and the plates were incubated at 35 °C for 24 h. The MBC was considered the lowest concentration of the compounds causing no visible growth on BHI medium. The MBC/MIC ratio was calculated to determine if the compounds have bacteriostatic (MBC/MIC ≥ 4) or bactericidal (MBC/MIC <4) properties [23]. As a result of this assay, we selected the *S. aureus* (ATCC 25923) and *K. pneumoniae* (ATCC 70603) bacterial strains to proceed with further assays.

2.10. Checkerboard microdilution assay

The evaluation of synergism between EpTI and the standard antimicrobial against *S. aureus* (ATCC 25923) and *K. pneumoniae* (ATCC 70603) bacterial was verified through the 'checkerboard assay'. The susceptibility test was developed using the microdilution method in broth according to the standards established by CLSI (M27-A6), which consisted of the application of two substances (EpTI and antimicrobial) on the microorganism with the lowest MIC, consequently, determining the MIC of the substances together. The culture medium used was Muller Hinton. The result was evaluated through the lowest concentration of each substance capable of completely inhibiting growth. For this, the Fractional Inhibitory Concentration Index (ICIF) was used, which corresponds to the addition of the Fractional Inhibitory Concentrations (CIF). These are equivalent to the ratio of the MIC of substances A and B combined by the MIC of each substance, as described below. CIFA = MIC of substance A with B / MIC of A alone; CIFB = MIC of substance B with A / MIC of B alone; ICIF = CIFA + CIFB. All experiments were carried out in triplicate in independent experiments. [24].

2.11. Crystal Violet uptake assay

To investigate whether EpTI affects the integrity of the bacterial plasma membrane, we used the crystal violet uptake assay. EpTI was incubated with *S. aureus* (ATCC 25923) and *K. pneumoniae* (ATCC 70603) at the MIC for each strain. The bacterial culture was incubated overnight in BHI. The bacterial suspension was adjusted to 0.5 on the McFarland scale. Then 180 μL of the bacterial suspension was added to 20 μL of the EpTI at MIC. The negative control contained 180 μL of bacterial suspension and 20 μL of 0.9% saline. After incubation at 37 °C for 4 h, the bacteria were collected by centrifugation at 9300 x g for 5 min and resuspended in saline containing 10 μg ml^{-1} of crystal violet. Sample and control were incubated at 37 °C for 10 min. After this time, the suspension was centrifuged at 13,400 x g for 15 min. The supernatants were collected, and the absorbance read at 570 nm [25,26], as well the absorbance of crystal violet solution. The experiments were carried in triplicate in three independents assays. For the calculation of the uptake percentage, the crystal violet solution was considered as 100%. The crystal violet uptake value by the sample and the control was calculated using the following formula:

Crystal violet uptake = (Abs sample/Abs crystal violet solution) x 100

2.12. Inhibitory effects on adhesion to polystyrene (in vitro)

In vitro adhesion assay was performed following the methodology of Emeri [27] with modifications. EpTI, ciprofloxacin, and vancomycin were diluted in ethanol at MIC/2. Then 200 μL of the compounds were added to a polyethylene microplate and maintained therein for 4 h in a laminar flow chamber for solvent evaporation. Suspensions of *K. pneumoniae* and *S. aureus* (1×10^7 $CFU.ml^{-1}$) were prepared in brain

heart infusion (BHI) and a 100 μL aliquot was added into the pre-treated microplate wells and allowed to remain in contact for 2 h at 37 °C for cell adhesion. The supernatant was removed, the wells were washed with 0.9% saline, and the remaining adhered cells were plated for CFU number count. The control with ethanol was added to show that the diluent does not influence cell adhesion.

2.13. Inhibitory effects of EpTI on biofilm formation

To study the inhibition of formation and preformed biofilm, the strains *S. aureus* (ATCC 25923) and *K. pneumoniae* (ATCC 70603) were used. An aliquot of 100 μL standardized cell suspension (1×10^8 CFU. ml^{-1}) was added to 96-well microplates. The microplates were incubated at 37 °C for 2 h to allow for cell adherence. The wells were washed with 0.9% saline to remove non-adherent bacterial, and then, the adhered cells were treated with EpTI at MIC and 10 x MIC for 24 h at 37 °C. Then, the microplates were washed to remove planktonic and killed cells, and then serially diluted and plated for CFU measurement. Vancomycin and ciprofloxacin were used as a standard drug, and negative control with culture medium alone was included [27].

2.14. Effects of EpTI on preformed biofilms

A total of 100 μL bacterial inoculum (1×10^8 CFU.ml^{-1}) was added to BHI plus 0.1% glucose (BHI-g) and then, the microplates were incubated at 37 °C for 24 h to allow for biofilm formation. Followed the biofilm formation, the wells were washed three times with 0.9% saline to remove planktonic cells. Then, 100 μL BHI-g containing the EpTI (MIC and 10 x MIC) was added. Vancomycin and ciprofloxacin were used as a standard drug (MIC and 10XMIC) and negative control with culture medium alone was included. The microplates were incubated at 37 °C for 24 h. After the incubation period, the microplates were washed with 0.9% saline and the counting of CFU.ml^{-1} was determined [27].

2.15. Quantification of biofilm cells

The biofilms were detachment off the wells using a 100 μL micropipette to allow dissociation of the cells. A volume of 50 μL containing the cell suspension was aspirated from the wells, transferred to a tube containing 450 μL of 0.9% saline and subjected to agitation. Ten Serial dilutions were performed and 100 μL of each suspension were plated onto Brain Heart Infusion Agar (BHI) culture medium plates to facilitate counting of bacteria. The survival percentage (CFU – colony forming unit) was determined based on the comparative survival of untreated biofilms [27].

2.16. Toxicity of EpTI in Galleria mellonella model

This assay was performed to evaluate the acute toxic effects of EpTI as described by Megaw et al. [28]. Ten *G. mellonella* larvae were randomly selected for each group weighing 0.2–0.3 g without signs of melanization. Ten microliters of EpTI in different concentrations (100, 50, 10, and 5 μM) were injected into the hemocoel of each larva through the last left proleg using a Hamilton syringe. The larval survival was recorded at selected intervals for 96 h. The larvae that did not show movement upon touch or with high levels of melanization (analyzed visually - darkened) were considered dead [3].

2.17. Statistical analysis

All statistical analyzes were performed using GraphPad Prism. All data were submitted to Shapiro–Wilk test to check for normal distribution. The data concerning the biofilm assays were analyzed by one-way analysis of variance (ANOVA) with Tukey's multiple comparison test, with a 5% significance level. For the *G. mellonella* toxicity model, Kaplan–Meier killing curves were plotted on GraphPad Prism 5.0 (GraphPad Software) and estimations of differences in survival were compared using the log-rank test.

3. Results

3.1. Purification and characterization of EpTI

We combined two chromatography steps, ion-exchange and bio-affinity, to obtain EpTI at a high purity level. In the initial step using a DEAE-Sepharose column, four peaks were collected. Among the peaks, two peaks were obtained during the elution step with a linear gradient of NaCl, D-III, and D-IV (Fig. 1A). Assays of inhibitory activity against trypsin were carried out with all peaks collected. The peak D-III showed the major inhibitory activity. For this reason, was chosen to the next chromatographic step. Then, the fraction D-III was applied on a trypsin-Sepharose column (Fig. 1B). During the washing step a first peak (S-I) was collected, while the use of HCl (50 mM) yield a single peak from the column (S-II). Once that S-II showed inhibitory activity against trypsin, this peak was named EpTI. The purification process was accompanied by 15% SDS-PAGE (Fig. 2A), where we observed that the fraction D-III showed a higher concentration of EpTI in comparison with the CE. The analysis of EpTI, from the trypsin-Sepharose column, revealed the occurrence of three protein bands with similar molecular weight, from 17 to 20 kDa, suggesting an apparent molecular heterogeneity.

To determine whether the three bands display inhibitory activity, we carried out negative staining using the substrate APNE and trypsin. In Fig. 2B three clear bands against a pink background were visible, ranging from 17 to 20 kDa, indicating that the hydrolysis of APNE substrate by trypsin was inhibited. This result confirms the occurrence of three proteins with different molecular weight which shown inhibitory activity against trypsin (Table 1).

Finally, to verify if the three proteins are different trypsin inhibitors, we carried the amino-terminal sequencing of the three bands (Table 2). The sequencing was submitted to NCBI-BLAST and compared with other PIs sequences to determine the homology of purified bands. The first 30

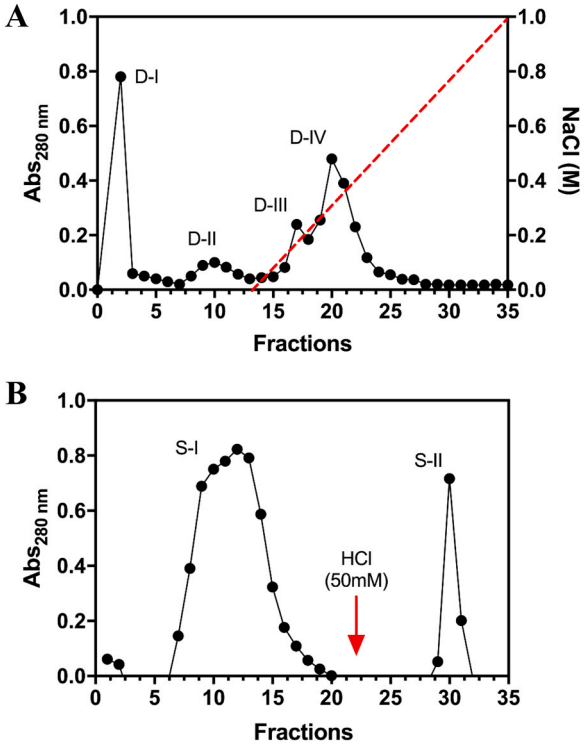

Fig. 1. Purification of EpTI. (A) DEAE-Sepharose, and (B) trypsin-Sepharose profile of separation to obtainment of EpTI.

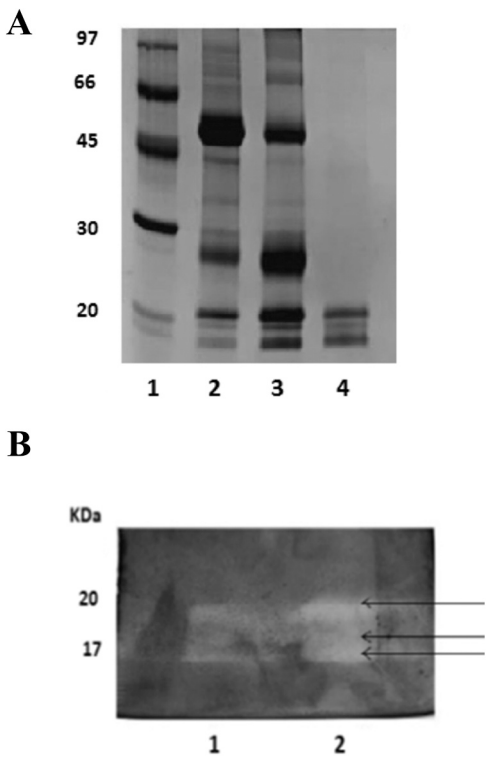

Fig. 2. Purification of EpTI. (A) SDS-PAGE 15%: (1) Molecular weight marker; (2) Crude extract; (3) D-III DEAE-Sepharose; (4) S-II Trypsin-Sepharose. (B) Negative staining of EpTI with APNE/TETRA and incubation with trypsin. White areas indicate inhibition of trypsin by 2.5 μg (1) and 5 μg (2) of EpTI.

amino acids from three proteins were sequenced and shared an identical sequence, demonstrating that the bands are, in fact, isoinhibitors, highly similar proteins that originate from a single gene or gene family and are the result of genetic differences. The comparison between EpTI sequence and other plant PIs revealed homology with Kunitz inhibitors (Table 2).

The stability of EpTI against thermal and pH ranging was evaluated (Fig. 3). The inhibitory activity was unchanged up to 100 °C (Fig. 2A). Regards to pH, acid values (from 2 to 5) prompted a reduction in inhibitory activity. Finally, the contribution of disulfide bonds in the stabilization of EpTI was investigated. Higher DTT concentrations (10 and 100 mM) were able to reduce the inhibitory activity of EpTI in all intervals (Fig. 2C), demonstrating the occurrence of disulfide bonds. The inhibitory activity of EpTI was more affected from 120 min at the highest DTT concentration (100 mM) (Fig. 2C). This result suggests that the time and concentration of DTT are determinants of EpTI inhibitory activity.

To determine the inhibition stoichiometry for trypsin, curves with different molar ratios between EpTI and trypsin were assayed. The complete inhibition of trypsin was obtained at a 1:1 molar ratio EpTI: trypsin (Supplementary Material S1). The enzyme kinetics model showed that the initial velocity parameters measured were plotted against a range of inhibitor concentrations, from 0.10 to 1.0 nM. The calculated values for Km and dissociation constant (Ki) were 0.49 nM and 13.1 nM, respectively. The stoichiometric studies showed that EpTI possesses a single reactive site for trypsin.

3.2. Antibacterial activity against planktonic cells

The MIC values of EpTI ranged from 5 to 10 μM against *E. aerogenes*, *K. pneumoniae*, *S. aureus*, and *S. haemolyticus* (Table 3), demonstrating that EpTI possesses antimicrobial activity. The MBC/MIC ratio was a shred of evidence that EpTI possesses predominantly bactericidal activity against these strains. The ratio MFC/MIC found for the EpTI demonstrated a bactericidal effect of these samples against the majority of the bacteria species tested, causing irreversible damage to the bacteria membrane.

3.3. Checkerboard microdilution assay

The type of interaction between EpTI and antibiotics was investigated whether antagonistic, additive, synergistic, or indifferent. The values of ∑FIC is shown in Table 4. *K. pneumonie* and *S. aureus* were chosen as representative of Gram-negative and Gram-positive bacteria, respectively. For *K. pneumoniae* strains there was synergistic interaction between EpTI and ciprofloxacin (∑CIF 0.3) whereas, for *S. aureus*, the combination of EpTI and vancomycin was classified as additive (∑CIF 0.9).

3.4. Crystal violet uptake assay

To investigate the EpTI effects on bacterial plasma membrane, we carried out experiments using the crystal violet uptake. The incubation of EpTI with *S. aureus* and *K. pneumoniae* strains at MIC for 10 min,

Table 1
Purification steps of *E. poeppigiana* trypsin inhibitor.

Steps	Total protein (mg)	Total activity (IU)[a] × 10^6	Specific activity (IU mg^{-1}) x 10^3	Purification (Fold)	Yield (%)
Crude extract	2000	17.55	8.77	1	100
DEAE-Sepharose	97.76	2.30	24.04	2.74	13.10
Trypsin-Sepharose	9.57	0.78	81.96	9.34	4.44

[a] One Inhibition Unity (IU) was defined as the amount of inhibitor that decreased the absorbance at 410 nm by 0.01 O.D. using the tripsin inhibition assay.

Table 2
Alignment of the N-terminal sequences of Kunitz inhibitors with the highest identities to EpTI.

```
EpTI          --LLPGGGXVIVNGGTYYLLLPQVXA-DGGDGL
DE-3          V-LLDGNGEVVQNGGTYYLL-PQVWA-QGGGVQ
ECI           EPLVDLEGNLVENGGTYYLL-PHIWA-LGGGIE
ETIB          E-LVDVEGEDVVNGGTYYML-PGIEG-DGGGME
PI_A_like     --VTDRDGDALRNGGTYHIL--PLFGVKDGGIE
WBTI-2        -ELVDVEGKTVRNGGTYYLV-PQLRP-GGGGME
                :    *  : *****:::   :    .*.
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Trypsin inhibitor from *Erythrina caffra (DE-3), Erythrina variegata var. Orientalis (ECI), Erythrina variegata (ETIB), Cajanus cajan (PI A-like) and Psophocarpus tetragonolobus (WBTI-2). The alignment was carried out in T-COFFEE Multiple Sequence Alignment Server.*

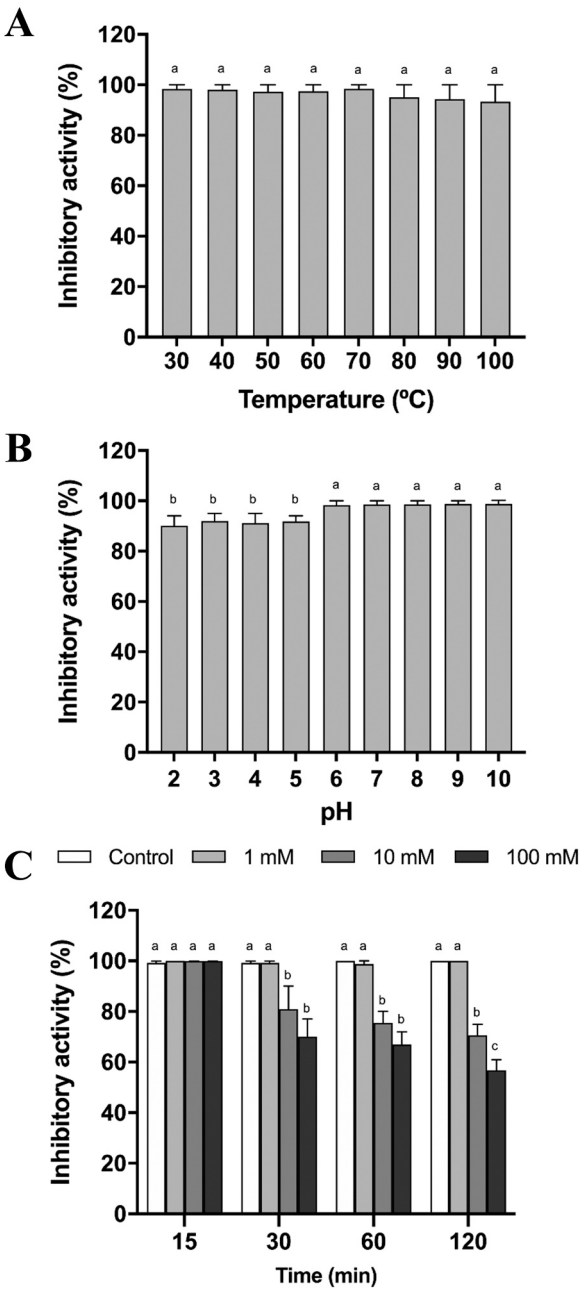

Fig. 3. Physicochemical features of EpTI. (A) Thermal stability was measured following an incubation period of 30 min and (B) pH stability after incubation at the indicated pH for 1 h at 30 °C. (C) Effect of DTT on the inhibitory activity of EpTI at different concentrations (1, 10, and 100 mM) and incubation times (15–120 min). Different letters indicate significant differences ($p < 0.05$).

increased the crystal violet uptake by 30% and 50%, respectively, when compared to the control (Fig. 4). The increase in the crystal violet uptake suggests that damages to bacterial plasma membrane occurred followed by EpTI incubation.

3.5. Inhibitory effects on adhesion in vitro

Studies on bacterial adhesion are important since the adherence of bacteria to biotic or abiotic surfaces facilitates the colonization of various niches. We investigated the effects of EpTI on bacterial adhesion, using the antibiotics vancomycin and ciprofloxacin. Fig. 5 shows that the EpTI treatment at MIC/2 decreased the adhesion of *S. aureus* and *K. pneumoniae* by 35% and 45%, respectively. The reduction of adhesion

Table 3
Antimicrobial activity of EpTI.

Microorganisms	EpTI (MIC, μM)	EpTI (MBC, μM)	Ciprofloxacin (MIC, μM)	Vancomicin (MIC, μM)	MBC/MIC ratio (EpTI)
Acinetobacter baumannii ATCC 19606	> 20	–	48.28	–	–
Enterobacter aerogenes ATCC 13048	5	10	6.03	–	2
Enterobacter cloacae ATCC 13047	10	10	6.03	–	1
Escherichia coli ATCC 35218	> 20	–	0.12	–	–
Klebsiella pneumonia ATCC 70603	5	10	3.01	–	2
Proteus vulgaris ATCC 8424	> 20	–	1.50	–	–
Serratia marcescens ATCC 51741	> 20	–	6.03	–	–
Salmonella enterica ATCC 13880	> 20	–	15.08	–	–
Staphylococcus aureus ATCC 25923	10	10	–	0.68	1
Staphylococcus haemolyticus ATCC 29453	5	10	–	0.34	2
Staphylococcus saprophyticus ATCC 29970	> 20	–	–	0.34	–

The results are interpreted as bacteriostatic (MBC/MIC ≥4) or bactericidal (MBC/MIC < 4); MIC: Minimum inhibitory concentration MBC: Minimum bactericidal concentration.

prompted by EpTI was similar to observed for vancomycin, while EpTI was more effective than ciprofloxacin when compared to the untreated group.

3.6. Effects of EpTI on biofilm formation and mature biofilm survival

Since EpTI showed bactericidal effects, we investigated the effect of EpTI on the biofilm formation and eradication of mature biofilms. The strains selected were *S. aureus* (ATCC 25923) and *K. pneumoniae* (ATCC 70603). In this assay, EpTI and the antibiotics were used in two concentrations, 1x MIC and 10 x MIC. Regards biofilm formation, EpTI caused a decrease of 38% and 66% for *S. aureus* and 40% and 74% for *K. pneumoniae* biofilm formation at 1 x and 10 x MIC, respectively (Fig. 6A and B). The MIC of EpTI, ciprofloxacin, and vancomycin used in this assay are provided in Table 3. Furthermore, the inhibition of biofilm formation observed upon treatment with vancomycin or ciprofloxacin for *S. aureus* and *K. pneumoniae* were similar to EpTI (Fig. 6A and B). Preformed biofilms treated with EpTI and antibiotics at 10 x MIC prompted a reduction of 50% for *S. aureus* and 59% for *K. pneumoniae* (Fig. 6C and D). However, at 1 x MIC, it did not affect the biofilm activity of *S. aureus* ($p > 0.05$). These findings support the view that the inhibitory effects of EpTI on biofilm formation and survival are comparable to or more effective than those of the standard drug (Fig. 6).

3.7. Systemic toxicity of EpTI in Galleria mellonella model

Toxicity of EpTI was assessed using the *G. mellonella larvae*, to verify acute toxicity *in vivo*. As observed in Fig. 7, the larvae treated with EpTI presented low mortality. The viability at the highest EpTI concentration was ~80% ($p > 0.05$), suggesting a low cytotoxicity.

Table 4
Comparison of MIC of isolated and associated substances, FIC values (Fractional Inhibitory Concentration) and pharmacological effect of the combination of drugs against *Staphylococcus aureus* and *Klebsiella pneumoniae*.

Strain	Combination	Individual MIC		Combination MIC		∑FIC	Activity
		a	b	a	b	FIC_A+FIC_B	
S. aureus (ATCC 25923)	EpTI (a) + vancomycin (b)	10	0.34	5	0.15	0.9	Additive
K. pneumoniae (ATCC 70603)	EpTI (a) + ciprofloxacin (b)	5	3.0	0.3	0.3	0.16	Synergic

The values of ΣFIC are classified according to the ranges described in Doern, (2014).

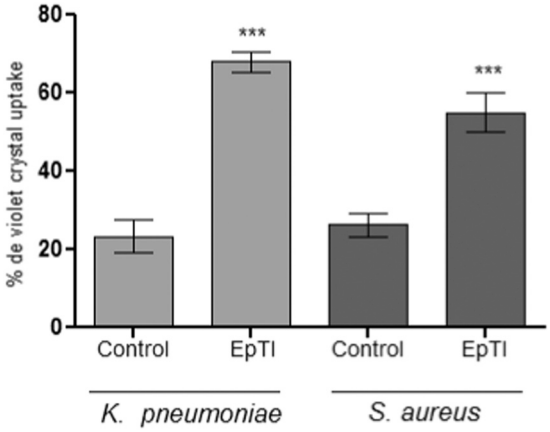

Fig. 4. Capture of crystal violet by *S. aureus* and *K. pneumoniae* exposed to EpTI. Values represent the means of three independent assays. Absorbance assessment read at 570 nm. Different letters indicate significant differences ($p < 0.05$).

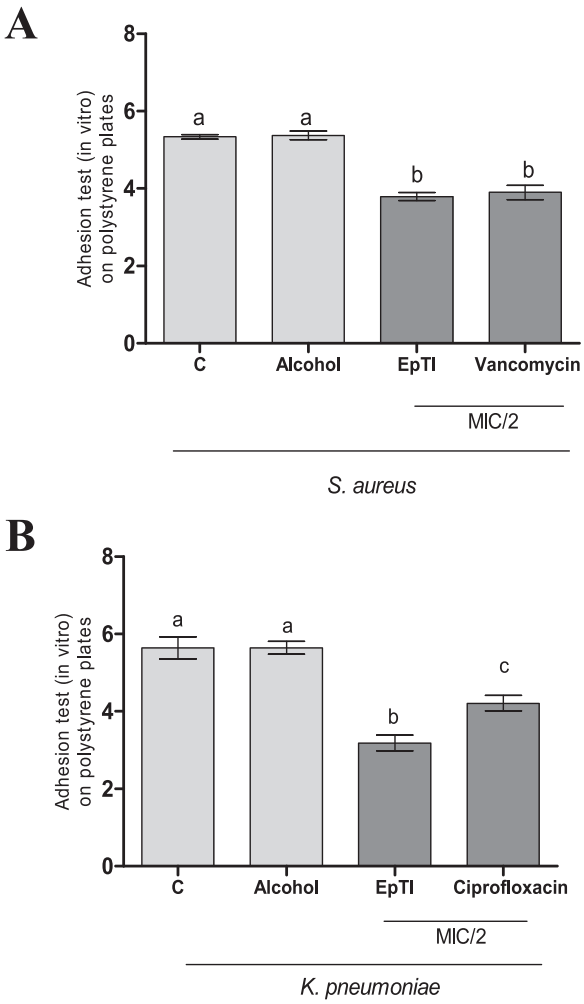

Fig. 5. Inhibitory effects (mean ± SD) of EpTI (A) *S. aureus* and (B) *K. pneumoniae* adhesion in polystyrene. Treatment with MIC/2 led to a significant decrease in adhesion to polystyrene compared to the untreated group. Different letters indicate significant differences ($p < 0.0001$, ANOVA with Tukey's post-test).

4. Discussion

We proposed to characterize a new PI from *E. poeppigiana* seeds, and to evaluate its antibacterial properties. To purify EpTI, two chromatography steps were combined, using ion-exchange and bioaffinity. Trypsin immobilized in agarose matrix successfully yield EpTI, used in further assays. EpTI purification process showed a yield of 4.44%, and was purified 9.34 fold, a higher purity than other Kunitz inhibitors such as PeTI (*Platypodium elegans* trypsin inhibitor) purified 4.40 fold and a yield of 3.40% [26]; a contrast yield whether compared with EvTI, (*Erythrina velutina* trypsin inhibitor), which presented a purification degree at the final step of 3200 times with an average recovery of 0.15% of purified [29].

Analysis of SDS-PAGE of EpTI revealed the presence of three polypeptide chains with apparent molecular weights of 20, 19, and 17 kDa (Fig. 1C). This apparent molecular weight is consistent with Kunitz inhibitors (from 18 to 26 kDa), such as EATI (*Entada acaciifolia* trypsin inhibitor) [29] ECTI (*Enterolobium contortisiliquum* trypsin inhibitor) [31,32] ILTI [15], and of the EvTI, which has a molecular mass of 17 kDa [29].

Negative staining of proteins was carried and demonstrated the presence of three isoinhibitors. The occurrence of isoinhibitors is a common feature shared by plant PIs. Similarly, in *Acacia victoriae* seeds [33], the three proteins with different molecular weight inhibited trypsin and were considered isoinhibitors. EATI, another Kunitz inhibitor, is composed of four isoinhibitors [30]. The occurrence of isoinhibitors is common among PIs and has been reported in *C. cajan* (C11PI) [34] *Archidendron ellipticum* [35], and in *E. velutina* [36].

To confirm the occurrence of isoinhibitors, the three protein bands from SDS-PAGE were submitted to the amino-terminal sequence through Edman degradation. Even sharing different molecular weight, the three proteins showed identical amino-terminal sequence. Furthermore, NCBI-BLAST revealed homology of EpTI with Kunitz inhibitors, sharing a conserved motif NGGTYY (Table 2). The similarity in EpTI amino-terminal sequence suggests that EpTI isoinhibitors origin from a single gene or gene family and are the result of post-translational modifications, explaining the differences in molecular weights. PIs are mainly accumulated in seeds and tubers and display an increased expression in vegetative parts affected by herbivores. During the coevolution between insects and plants, physiological and biochemical mechanisms led plants to develop PIs with special characteristics, such as the increased complexity of existing inhibitors, in turn producing highly specific isoforms [33].

EpTI maintained a high inhibitory activity upon a high range of pH and temperature, a common feature shared among Kunitz inhibitors [37]. EvTI maintains its inhibitory activity under similar conditions, and PDTI (*Peltophorum dubium* trypsin inhibitor) maintains its activity at

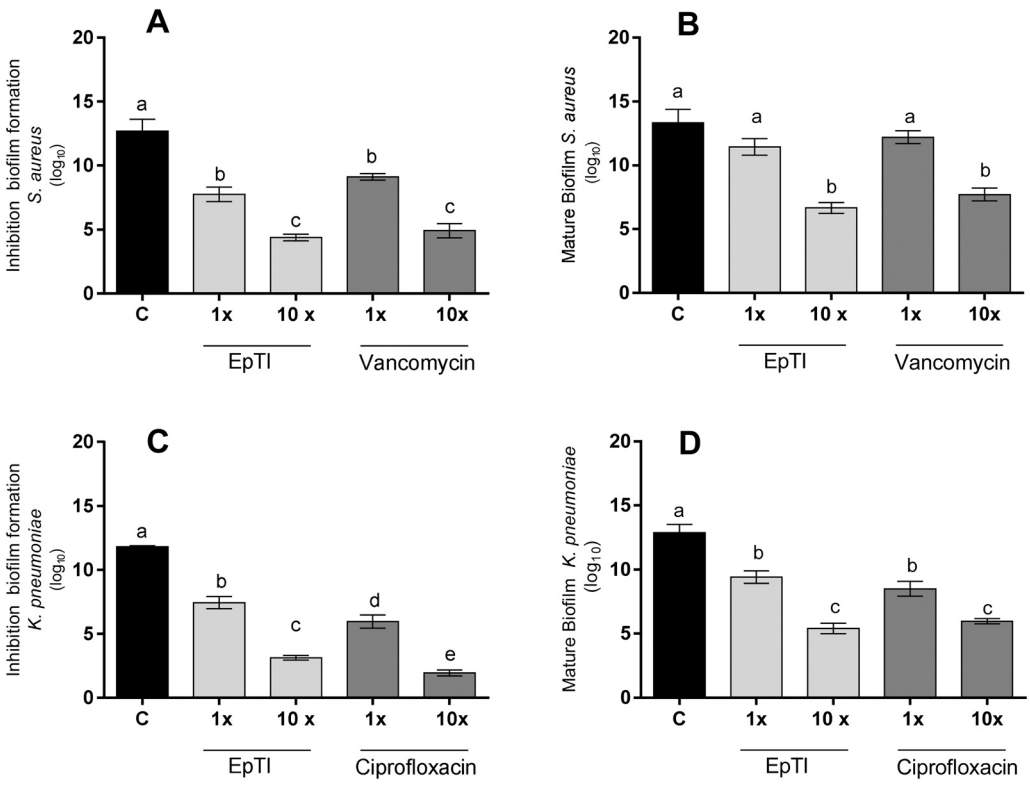

Fig. 6. Quantitative analysis (determination of CFU ml^{-1}) of the inhibitory effects (mean ± SD) of EpTI (A and B) *S. aureus*, (C and D) *K. pneumoniae* biofilm formation and mature biofilm survival. Treatment with EpTI, vancomycin, and ciprofloxacin at 10xMIC caused a significant decrease in *S. aureus* and *K. pneumoniae* biofilm formation compared to the untreated group ($p < 0.0001$, ANOVA with Tukey's post-test). Quantitative analysis of mature biofilms treated with EpTI, vancomycin, and ciprofloxacin showed that 10xMIC all compounds led to a significant decrease in *S. aureus* and *K. pneumoniae* biofilm survival compared to the untreated group ($p < 0.0001$, ANOVA with Tukey's post-test).

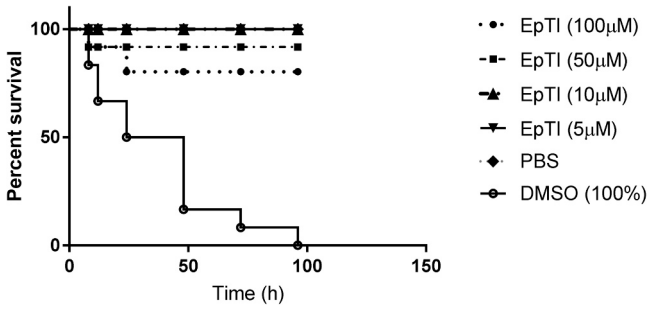

Fig. 7. Survival percentage over time of *Galleria mellonella* larvae treated with EpTI which correspond to their effective antibiofilm concentrations previously determined ($p < 0.05$, log-rank test).

varying pH and temperatures of up to 100 °C [37]. Two isoinhibitors from *Pithecellobium dumosum* inhibitor, JB2, and JB4, also maintain their stability after incubation at 30–100 °C [39] and the *Poecilanthe parviflora* trypsin inhibitor (PPTI) exhibits maximum inhibitory activity after incubation at pH 2–10 for 60 min [39].

The high stability of Kunitz inhibitors can be explained due to the presence of cysteine residues along the molecule, forming a variable number of disulfide bonds. This covalent binding is responsible for maintaining overall folding, establishing the conformation of the reactive site, and preserving its inhibitory activity [40,41]. Some PIs present similar characteristics of high stability, such as ILTI [11] and *P. roxburghii* (PrTI) [42]. Since the disulfide bonds are essential for inhibitory activity in Kunitz inhibitors, we evaluated the effect of the reducing agent DTT on EpTI inhibitory activity. Interestingly, DTT exposure showed a time-dependent and concentration-dependent effect. Within 30 min of exposure, no reduction in inhibitory activity was observed, while longer exposure (60 and 120 min) prompted reduction on the inhibitory activity proportional to the DTT concentrations (Fig. 2C). PrTI maintains a high inhibitory activity even in the presence of DTT [41]. Similar results have been observed for PIs from *E. caffra* [43]. Kunitz inhibitors are known to have a variable number of disulfide bonds and these bonds, when reduced, may influence the functionality of PIs since the reactive sites may be maintained by this covalent binding or by weak interactions such as those provided by hydrogen bonds [18]. As the functional structure of EpTI was impaired by the DTT incubation, we suggest that the disulfide bonds promote significant stabilization of the overall folding of EpTI, especially at its reactive site [44].

Additionally, stoichiometric studies showed that EpTI inhibits trypsin at the molar ratio of 1:1 (enzyme: inhibitor), demonstrating the presence of a single reactive site for trypsin (Fig. 3), similar to that of the *Adenanthera pavonina* trypsin inhibitor (ApTI) [45] and *Poincianella pyramidalis* trypsin inhibitor (PpyTI) [46]. The dissociation constant (Ki) was calculated as 13.1 nM (Fig. 4), similar to that observed in *E. velutina* (EvTIb, 10 nM) [29], but higher than reported for ILTI (6 nM) [11], ApTI (4.46 nM) [46], and *Tamarindus indica* trypsin inhibitor (TTI, 1.7 nM) [47]. Most Kunitz inhibitors are competitive and form highly stable enzyme-inhibitor complexes, with Ki values ranging from 10^{-8} to 10^{-13} M. Based on the parameters of Vmax and Km, it was possible to infer that EpTI displays a similar inhibition mechanism shared with most Kunitz inhibitors.

Antibiotics possess great importance in treatment of infections, but the natural selection and their overuse and misuse have contributed to increasing antibiotic resistance worldwide, which in turn leads to higher morbidity, mortality, disease prolongation, reduced work efficiency, and increased hospitalization costs [48]. Given this problem, WHO has created a global priority list of antibiotic-resistant organisms to guide the research, discovery, and development of new antibiotics. From this list, *S. aureus* and *K. pneumoniae* were related to human infections. According to the Infectious Diseases Society of America (IDSA), the most worrying bacteria comprise the multidrug-resistant pathogens *E. faecium*, *S. aureus*, *K. pneumoniae*, *A. baumannii*, and *Enterobacter species* (ESKAPE), that together are responsible for the majority US hospital infections. Furthermore, resistance to last-generation antibiotics is spreading rapidly [49].

PIs have an essential role in the treatment of various diseases such as cardiovascular disorders [50], cancer [51,52], and HIV [53]. Besides, PIs also have activity against fungi and bacteria [14]. We describe, for the first time, a PI isolated from *E. poeppigiana* with antibacterial and antibiofilm activities. Regarding antimicrobial activity, we observed that EpTI showed activity against several pathogens of medical importance (Table 3). Some PIs have similar antimicrobial activities; for example, the trypsin inhibitor JcTI-I, from *Jatropha curcas* seeds, reduces the growth of *S. aureus* and *S. enterica* but with a concentration higher than 25 μM [54]. However, EvTI, a PI isolated of a plant from the same genus that EpTI, was unable to inhibit the growth of *E. coli* and *S. aureus* [29]. EpTI increased the uptake of crystal violet. The greater passage of the dye through the bacterial plasma membrane indicates the commitment of membrane integrity.

To evaluate a possible interaction between EpTI and the antibiotics used in the treatment of systemic infections, the association of EpTI with vancomycin and ciprofloxacin was analyzed. The observed results were synergistic for *K. pneumoniae* and additive for *S. aureus*, both interpreted as a positive interaction, based in a reduction of MIC. The reduction in the MIC is clinically interesting because even at lower concentrations, both EpTI and antibiotics could still have an inhibitory effect on bacterial growth.

Bacterial binding to host tissues is one of the strategies for the success of infection [23]. Adherence may occur through hydrophobicity or adhesins, pili, or fimbriae [25,55]. EpTI inhibited polystyrene adhesion of *S. aureus* and *K. pneumoniae* at concentrations below the MIC. Inhibitory effects were more pronounced against *K. pneumoniae* (Fig. 3). These results may contribute to the prevention of medical device-related infections, once these devices would be pretreated with molecules able to reduce the bacteria adherence [27].

Bacteria associated with biofilm are highly resistant to antibiotics [56,57]. The effective concentration of antimicrobials against biofilms can reach 1000 higher than in planktonic cultures [58]. Our results showed that EpTI was able to inhibit the formation and to treat the mature biofilm form in both strains assayed. However, *S. aureus* was more resistant to drugs than *K. pneumoniae*. Although there are few studies on anti-biofilm activity with PIs, recently we showed that ILTI shows inhibitory activity against *S. aureus* and *S. epidermidis* biofilms [14]. Also, Kunitz PIs have been implicated in the modulation of host response during the development and proliferation of pathogens [52]. Thus, we conclude that EpTI has a marked antibacterial activity *in vitro* and displayed low toxicity in the *G. mellonella* model. Our results suggest that EpTI could act disrupting the cell membrane integrity, however, further studies are needed to confirm this hypothesis.

5. Conclusion

A new Kunitz inhibitor with anti-biofilm properties was purified from *E. poeppigiana* seeds, demonstrating the potential for the development of new antimicrobial agents. Drugs with a mechanism of action different from classical antibiotics are desirable to overcome the bacteria resistance against antibiotics. Once that a novel mechanism would be described, a new class of compounds could be designed. Is important to note that there are no drugs able to control bacterial biofilms. This feature encourages further studies using EpTI sequence as a template to design peptides and to investigate their anti-adhesive properties.

CRediT authorship contribution statement

KMAB, JCOS,CFRO and MLRM: Conception or design of the work, data analysis and interpretation, drafting of the article, and critical revision of the article. SMN, AJM, SRR, DGLO, GSP and SSW: data collection and data analysis and interpretation. The authors declare no conflicts of interest.

Conflict of interest statement

The authors declare no competing interests.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2021.112198.

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