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# PgTI, the First Bioactive Protein Isolated from the Cactus *Pilosocereus gounellei*, is a Trypsin Inhibitor with Antimicrobial Activity

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***Authors' contributions***

*This work was carried out in collaboration among all authors. Authors CAARF, EVP, THN and PMGP designed the study. Author THN performed the statistical analysis. Authors CAARF, RBZ, EVP, THN and PMGP wrote the protocol. Authors CAARF, PKA, EVP and THN wrote the first draft of the manuscript. Authors CAARF, PKA, TAL, PMS, MCM, RBZ, EVP and THN managed the analyses of the study. Authors CAARF, PKA, TAL and THN managed the literature searches. Authors EVP, LCBBC, THN and PMGP revised the final version of the manuscript. All authors read and approved the final manuscript.*

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## ABSTRACT

**Aims:** This work aimed to isolate, characterize and evaluate the antimicrobial activity of a trypsin inhibitor (PgTI) from the stem of *Pilosocereus gounellei*.
**Place and Duration of Study:** Departamento de Bioquímica, Universidade Federal de Pernambuco between March 2013 and October 2018. Instituto de Bioquímica Médica Leopoldo de Meis, Universidade Federal do Rio de Janeiro between June and July 2018.
**Methodology:** PgTI was isolated from *P. gounellei* stem extract by gel filtration and ion exchange chromatographies. The inhibitor was characterized by isoelectric focusing, polyacrylamide gel electrophoresis, tryptic digestion followed by mass spectrometry analysis and for stability towards heating. Antibacterial and antifungal activities were investigated through broth microdilution assays. Viability of the microbial cells was also evaluated by flow cytometry analysis using thiazol orange and propidium iodide.
**Results:** PgTI appeared as a single polypeptide band of 37.1 kDa and isoelectric point (pI) 5.88. The inhibition constant ($K_i$) for bovine trypsin was 14 nM and mass spectrometry analysis of PgTI did not reveal similarities with other plant proteins. Trypsin inhibitor activity was stable at temperatures up to 50ºC. PgTI inhibited growth of Gram-positive and Gram-negative bacteria (minimal inhibitory concentrations (MIC) from 7.5 to150 µg/mL) with bactericidal activity only against *Escherichia coli* (minimal bactericidal concentration: 75.0 µg/mL). PgTI also inhibited the growth of *Candida krusei* (MIC of 60 µg/mL). Flow cytometry confirmed that PgTI did not affect the viability of *E. coli* and *C. krusei* cells at the MIC.
**Conclusion:** This is the first report on a bioactive protein purified from *P. gounellei*, which provides biotechnological value to this cactus.


*Keywords: Xique-xique; protease inhibitor; antimicrobial activity; cactaceae.*

## 1. INTRODUCTION

Antibiotic resistance is a big concern for health care systems due to the possibility of the emergence of multiple infectious diseases with no viable therapy. Self-medication, indiscriminate prescribing and prolonged use are factors associated with resistance. In addition, overuse and/or misuse of antibiotics in the food industry and veterinary medicine has led to resistance [1]. The search for new antimicrobial agents is stimulated aiming at developing antibiotics to be used together or as substitutes for the current drugs.

Protease inhibitors (PIs) are molecules able to interact with an enzyme molecule to reduce or block its catalytic activity. In plants, they can be secondary metabolites, such as flavonoids, or proteins [2]. Proteinaceous PIs isolated from plants have been reported as insecticidal, anti-angiogenic, trypanocidal, and antimicrobial agents [3–8]. These inhibitors can affect replication or viability of microbial cells by inhibiting the activity of proteases or damaging the cell wall or plasma membrane leading to alteration of permeability [8].

The Caatinga region is an exclusive Brazilian plant formation that stands out due to its high diversity of plant species, many of which are employed in traditional medicine [9]. Arcoverde et al. [10] reported on the presence of trypsin inhibitor activity in 23 plants from Caatinga and considered these plants interesting materials for exploitation by the scientific community.

*Pilosocereus gounellei* (F.A.C. Weber ex K. Schum.) Byles & G.D. Rowley, popularly known as “xique-xique”, is a cactus endemic of Caatinga. Its use in folk medicine has been reported. An ointment prepared from the stem is used for treating inflammatory processes resulting from injuries [11] and preparations of xique-xique roots are used for treating prostate and urethra inflammations [12]. There are few scientific publications on the biotechnological potentials of *P. gounellei*. Sousa et al. [13] showed gastroprotective effects of *P. gounellei* stem and root ethanolic extracts and Oliveira et al. [14] reported that saline extract from the stem showed antinociceptive activity in mice.

In this study, we purified and characterized a trypsin inhibitor (PgTI) from the stem of *P. gounellei*. In addition, the antimicrobial activity of PgTI against medically important bacteria and fungi was evaluated.

## 2. MATERIALS AND METHODS

### 2.1 Plant Material

Stem (columnar cladode) of *P. gounellei* was collected in Limoeiro, Pernambuco, Brazil. The authors have authorization (no. 38690) from the *Instituto Chico Mendes de Conservação da Biodiversidade* (ICMBio) from the Brazilian Ministry of the Environment for plant collection. The access was recorded (AE65D9B) in the *Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional. Associado* (SisGen). A voucher specimen (no. 82,853) was deposited at the Herbarium UFP Geraldo Mariz from the *Universidade Federal de Pernambuco*. The thorns were removed, and the stems were cut into small pieces and dried at 28°C for 3 days. Next, the material was powdered and stored at -20ºC.

### 2.2 Purification of PgTI

Stem powder was added to 0.15 M NaCl (5% w/v) and the mixture was stirred for 16 h at 28°C. After centrifugation (3,000 ×*g*, 15 min, 28ºC), the supernatant (saline extract) was loaded (2 mL) onto a Sephadex G-100 column (33 × 1 cm) equilibrated with 0.15 M NaCl at a flow rate of 6.0 mL/min. Fractions of 2 mL were collected and absorbance at 280 nm was monitored. Pooled fraction tubes 16–28 (P1) were evaluated for protein concentration and trypsin inhibitor activity as described below. P1 was dialyzed at 28ºC for 4 h in a 10-kDa cut-off membrane against 0.1 M Tris-HCl pH 8.0 and 2.0 mL (2.0 mg of protein) was loaded onto an ion exchange DEAE FF 16/10 column coupled to the ÄKTAprime system (GE Healthcare Life Sciences, Sweden). The column was equilibrated with 0.1 M Tris-HCl pH 8.0 at a flow rate of 5.0 mL/min. After equilibrating the column, adsorbed proteins were eluted with Tris buffer containing 1.0 M NaCl. Eluted fractions with an absorbance higher than 0.100 were pooled, dialyzed against distilled water for 4 h and dried by lyophilization. Purification yield corresponded to the amount of protein from P1 recovered following purification. For determination of trypsin inhibitor activity and dissociation constant ($K_i$), PgTI was suspended in 0.1 M Tris-HCl pH 8.0. For the antimicrobial assays, PgTI was suspended in distilled water.

### 2.3 Protein Concentration and Trypsin Inhibitor Activity

Protein concentration was estimated according to Lowry et al. [15] using bovine serum albumin (31.25–500 µg/mL) as the standard. Trypsin inhibitor activity was evaluated using 0.1 mg/mL bovine trypsin and the substrate *N*-benzoyl-DL-arginyl-ρ-nitroanilide (BApNA) as described by Pontual et al. [4]. Bovine trypsin (5 µL) was incubated for 5 min at 37ºC with aliquots of PgTI (30 µL). The total volume was adjusted for 200 µL with 0.1 M Tris-HCl pH 8.0 containing 0.02 M $CaCl_2$. Next, 8 mM BApNA (5 µL), dissolved in dimethyl sulfoxide (DMSO), was added to the mixture and incubated for 30 min at 37ºC. A control (100% of substrate hydrolysis) reaction between trypsin and BApNA was run in absence of PgTI. Substrate hydrolysis was followed by measurement of absorbance at 405 nm. Blank reactions were performed under the same conditions, without substrate or enzyme. One unit of trypsin inhibitor activity was defined as the amount of inhibitor that decreases the absorbance by 0.01 after 30 min at 37°C compared to control. Specific activity was determined as the ratio between trypsin inhibitor activity (U) and protein concentration (mg).

### 2.4 Two-dimensional Electrophoresis

PgTI (250 µg) was resuspended in rehydration buffer [8 M urea; 2% (w/v) CHAPS; 1% (v/v) IPG buffer pH 3–10; 0.002% (w/v) bromophenol blue] and taken up passively into a 7 cm Immobiline DryStrip pH 3–10 linear gradient, (GE Healthcare Life Sciences, Sweden) during rehydration for 16 h at 25°C. Isoelectric focusing was performed on the Ettan IPGPhor III at 20°C according to the manufacturer's instructions. Next, the strip was washed three times with 50 mM Tris-HCl pH 8.8 containing 6 M urea, 30% (v/v) glycerol, 2% (w/v) sodium dodecyl sulfate (SDS) and 0.002% (w/v) bromophenol blue. The strip was then washed once with Tris-glycine-SDS buffer and transferred to a 12% (w/v) polyacrylamide gel containing SDS [16]. Standard molecular mass markers (12–225 kDa) were electrophoresed in the same gel. The gel was stained with 0.02% (w/v) Coomassie Brilliant Blue, destained, and analyzed using the ImageMaster software (GE Healthcare).

### 2.5 Mass Spectrometry (MS) Analysis

The PgTI protein spot was excised from the 2-dimensional gel and submitted to in-gel tryptic digestion as described by Pontual et al. [6]. MS/MS analysis was performed in electrospray ionization quadrupole time-of-flight (ESI-QUAD-TOF) mode and the peptide spectra were compared with sequences of Viridiplantae proteins (NCBInr database) using MASCOT

(http://www.matrixscience.com). Peptides matching with the contaminants keratin and trypsin were excluded. The analysis was performed in triplicate.

## 2.6 Determination of Inhibition Constant ($K_i$)

Dixon plot analysis was employed to determine $K_i$ for bovine trypsin [17]. Inhibition assays were performed using two BApNA concentrations (4 and 8 mM). Dixon plots were generated using the reciprocal velocity ($1/v$) versus inhibitor concentration and the intersection of the two regression lines for each BApNA concentration yielded the $K_i$.

## 2.7 Effect of Heating on Trypsin Inhibitor Activity

PgTI samples were heated in a water bath for 15 min at different temperatures (30–60°C). The heated PgTI samples were evaluated for trypsin inhibitor activity as described above. An unheated sample of PgTI was also evaluated.

## 2.8 Antimicrobial Activity

Bacterial strains (*Escherichia coli* ATCC-25922, *Enterococcus faecalis* ATCC-6057, *Micrococcus luteus* F00112, *Pseudomonas aeruginosa* UFPEDA-416, *Serratia* sp. UFPEDA-398, *Staphylococcus aureus* ATCC-6538 and *Staphylococcus saprophyticus* UFPEDA-833) were provided by the culture collection (WDCM 114) of the *Departamento de Antibióticos* from the *Universidade Federal de Pernambuco*, Recife, Brazil. Stored cultures of *Candida albicans* (URM 5901), *Candida parapsilosis* (URM 6345), and *Candida krusei* (URM 6391) were obtained from the culture collection University Recife Mycologia (URM), *Departamento de Micologia*, *Universidade Federal de Pernambuco*, Brazil. Bacteria were cultured in Mueller Hinton Agar (MHA) overnight at 37°C and yeasts were cultured in Sabouraud Dextrose Agar (SDA) overnight at 28°C. The densities of the microorganism cultures were adjusted turbidimetrically at a wavelength of 600 nm ($OD_{600}$) to $1\times10^8$ or $3\times10^6$ colony forming units (CFU) per mL in sterile saline (0.15 M NaCl).

Broth microdilution assays were performed to determine the minimum inhibitory concentration (MIC) values. First, 100 µL of sterile culture medium [Mueller Hinton Broth (MHB) for bacteria or Sabouraud Dextrose Broth (SDB) for yeasts] was added to all wells of a microplate. The first row served as a control for sterility and only contained culture medium. Next, 100 µL of PgTI (at a concentration of 0.6 mg/mL) was added to the third well, and a two-fold serial dilution was performed until the last well in that row. Finally, the microorganism suspension (10 µL at $10^8$ CFU/mL for bacteria or 20 µL at $3 \times 10^6$ CFU/mL for yeasts) was added to all wells except the first well. The second well, containing microorganism in the absence of PgTI, corresponded to the 100% growth control. Microplates were incubated at 37°C or 28°C for bacteria and yeasts, respectively. The $OD_{600}$ was measured at time zero and after 24 h using a microplate reader. MIC was defined as the lowest PgTI concentration able to reduce the optical density ≥ 50% compared with the 100% growth control.

For determination of the minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC), aliquots (10 µL) from the wells containing PgTI concentrations higher or equal to the MIC were inoculated into petri plates containing MHA or SDA, and incubated for 24 h at 37°C or 28°C. The MBC and MFC corresponded to the lowest concentration able to reduce the number of CFU by 99.9% compared to the initial inoculum. Each assay was conducted in triplicate and three independent experiments were performed.

## 2.9 Viability Analysis

The viability of microbial cells treated with PgTI was evaluated by testing membrane integrity using the Cell Viability Kit of BD Biosciences (San Jose, CA, USA). Isolates were incubated as described in the previous section with PgTI at the MIC. The negative control was prepared by adding distilled water instead of PgTI. For the positive control, cells were treated with 70% (v/v) isopropyl alcohol for 1 h before analysis. Samples were centrifuged (10,000 g, 10 min, 25°C) and the cell pellets were washed three times with 0.1 M PBS pH 7.0. Next, 42 µM thiazole orange (TO, 5 µL) and 4.3 mM propidium iodide (PI, 5 µL) were added to the samples, vortexed, and incubated for 5 min at 25ºC. After this, 50 µL of a fluorescent bead suspension (BD Liquid Counting Beads) was added, and the mixture was vortexed for 30 s. Data was acquired on a BD Accuri C6 cytometer (BD Biosciences) with an SSC threshold of 200 and stopped after gating 30,000 events for each

sample. Analysis was performed using the BD Accuri C6 Software. Results were presented as FL1 vs. FL3 dot plots and mean FL3 fluorescence (PI staining).

### 2.10 Statistical Analysis

Data were expressed as replicate means ± standard deviation (SD). One-way ANOVA (significance at $p < 0.05$) was conducted using Action 2.8.29.357.515 software (Estatcamp, Brazil). Significant differences between the treatment groups were analyzed using Tukey's test (significance at $p < 0.05$).

## 3. RESULTS AND DISCUSSION

The plant species, *P. gounellei*, is part of the underestimated biodiversity from Caatinga and was chosen to study in order to unravel the biochemical wealth of this region. Furthermore, the recognized biotechnological potential of protease inhibitors encouraged us to look at the presence of a bioactive protein from this class in *P. gounellei*.

The saline extract showed high viscosity, preventing the determination of protein concentration and trypsin inhibitor activity. Thus, we employed gel filtration chromatography to obtain a more workable preparation for protein purification. The chromatographic profile on Sephadex G-100 showed a single protein peak, which was deemed P1 (Fig. 1A). The high viscosity of the saline extract was most likely due to the high carbohydrate content of the plant tissue. Indeed, Nascimento et al. [18] reported that *P. gounellei* contains 5.7 g of carbohydrate per 100 g of stem, in contrast with a protein content of 0.4 g per 100 g. Oliveira et al. [14] reported the presence of sugars in a saline extract from this cactus using a similar methodology employed by us. Gel filtration chromatography was effective in decreasing the viscosity, due to separation of proteins from high molecular mass polysaccharides.

Chromatography of P1 on a DEAE FF 16/10 column resulted in a single peak of adsorbed proteins, eluted with 1.0 M NaCl (Fig. 1B). Eluted fractions were pooled (PgTI) and showed a specific activity higher than P1. Table 1 summarizes the results from the purification of PgTI. The high yield (98%) following the second chromatography step indicated that most of the proteins present in P1 bound to the ion exchange matrix.

Two-dimensional electrophoresis revealed PgTI as a single spot of 37.1 kDa and isoelectric point (pI) 5.88 (Fig. 1C. The pI value demonstrated the anionic nature of this protein, corroborating with the adsorption to an anion-exchanger (DEAE) matrix. Tandem mass spectrometry (MS/MS) of peptides derived from the in-gel tryptic digestion of the PgTI spot yielded 25 peptide matches (Table 2), but no similarities with Viridiplantae proteins were detected. Dixon plot analysis indicated a $K_i$ of 14 nM toward bovine trypsin. PgTI showed higher affinity for trypsin than inhibitors from *Moringa oleifera* flowers and *Tecoma stans* leaves ($K_i$ of 2.4 μM and 43 nM, respectively) but lower than inhibitors from *Entada acaciifolia* (1.75 nM) and *Enterolobium timbouva* (0.5 nM) seeds [4,19–21].

The trypsin inhibitor activity of PgTI was not significantly altered at temperatures up to 50°C, but was abolished at 60°C. Neutralization of trypsin inhibitor activity upon heating at 60°C may be due to denaturation of the PgTI domain responsible for inhibitory activity [22], as well as protein aggregation [23], preventing the interaction of the reactive sites with trypsin. Unlike PgTI, the activity of the trypsin inhibitor from *Senna tora* remained unchanged until 60ºC, and inhibitors from *Poincianella pyramidalis* and *Cassia grandis* were stable up to 70°C and 80ºC, respectively [24–26]. Despite this, PgTI still showed a relevant thermo-stability since its activity was not affected until temperatures ≥ 50°C.

The bacterial species evaluated here have medical relevance since they are known to cause pneumonia, infections of the digestive and urinary tracts, as well as more severe infections, such as endocarditis and bacteremia [27–31]. *C. krusei* is an emerging nosocomial pathogen primarily found in immunocompromised patients and those with cancer of hematologic-oncologic origin [32]. PgTI inhibited the growth of *E. coli*, *E. faecalis*, *M. luteus*, *P. aeruginosa*, *Serratia* sp., *S. aureus*, and *S. saprophyticus* with MIC values ranging from 7.5 to 150 μg/mL (Table 3). However, the inhibitor was bactericidal only against *E. coli* (MBC of 75 μg/mL). PgTI also only inhibited growth of *C. krusei* (MIC of 60 μg/mL) and no fungicidal effect was observed.

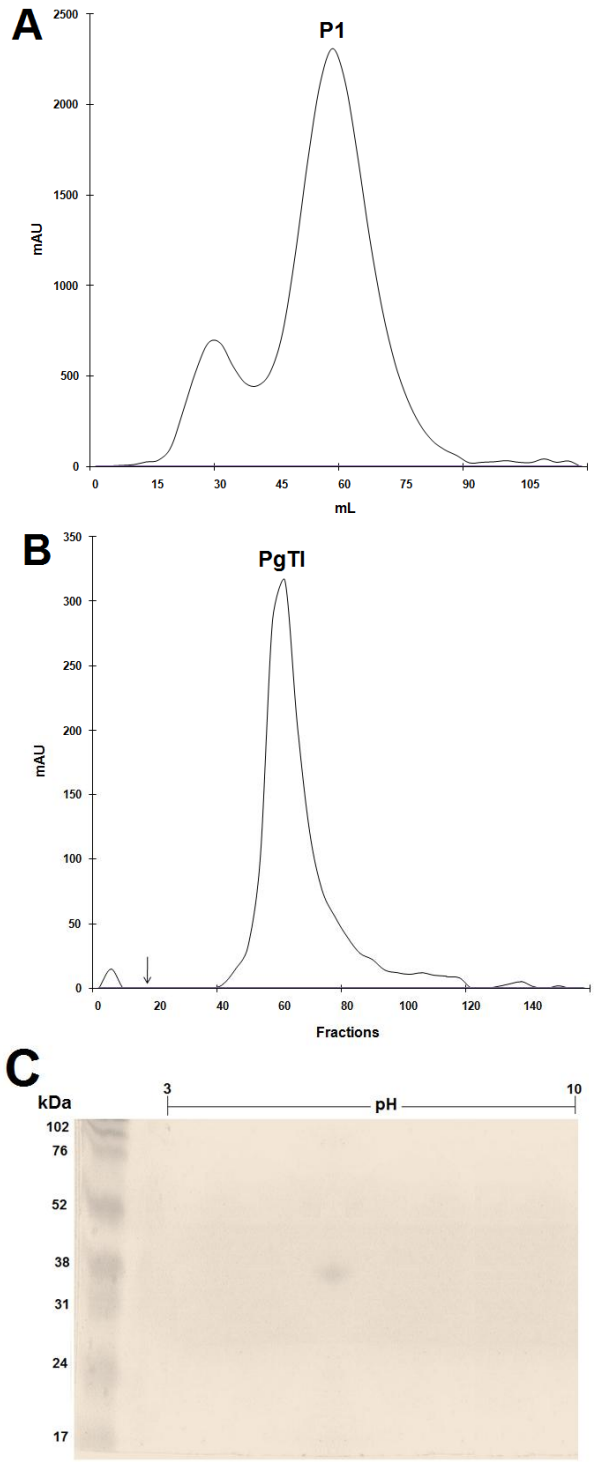


**Fig. 1. Purification of PgTI from the stem of *P. gounellei*. (A) Gel-filtration chromatography (Sephadex G-100) of *P. gounellei* stem extract in 0.15 M NaCl. (B) Ion exchange chromatography of P1 on a DEAE FF 16/10 column equilibrated with 0.1 M Tris-HCl pH 8.0 and eluted with 0.1 M Tris-HCl pH 8.0 containing 1.0 M NaCl. The arrow indicates the addition of eluent solution**

**Table 1. Purification of the trypsin inhibitor from *P. gounellei* stem extract (PgTI)**

| Sample | Total protein (mg) | Specific activity (U/mg) | Yield (%) | Purification factor[a] |
|---|---|---|---|---|
| P1 | 2.0 | 184 | 100 | 1 |
| PgTI | 1.96 | 373 | 98.0 | 2.02 |

*P1: protein peak recovered following chromatography of stem extract on Sephadex G-100. The purification corresponds to the ratio between the specific activity of PgTI and specific activity of P1*

**Table 2. Peptide matches and respective amino acid sequences obtained by MS/MS analysis of PgTI after in-gel tryptic digestion**

| Mass (Da) | Sequence |
|---|---|
| 823.4840 | VATAPLPR |
| 855.5104 | LATVSLPR |
| 865.4484 | AVASYLSR |
| 869.5266 | NGAGVSLPR |
| 880.5050 | RPGVVVVR |
| 898.5142 | GAAKGSVGPR |
| 912.5308 | GLDGVSIPR |
| 930.5044 | GSIVNINSK |
| 1021.5052 | KNMPLTYR |
| 1032.4990 | ITLESVDEK |
| 1089.5232 | TGLIEWEDK |
| 1101.5674 | QESPATLRSN |
| 1116.5630 | EFLDIDLPR |
| 1150.5890 | SVVTQAANYAK |
| 1163.5406 | QKYEELVQK |
| 1207.6102 | TQITKAAGPPPK |
| 1221.5856 | AVEENLKEYK |
| 1235.6012 | YGEEIKIEQK |
| 1321.6554 | ALGRLNPSYAMN + Oxidation (M) |
| 1358.7164 | SIGDIDSLLADLK |
| 1380.6306 | ALEESNYELEGK |
| 1421.6372 | MAAFSGQLAEQNR |
| 1437.6514 | VLMARNYMEAPK + Oxidation (M) |
| 1531.7936 | FANLEKNQVAQDR |
| 2267.0869 | EYSVELDVREWASDEEVGR |

**Table 3. Antimicrobial activity of the trypsin inhibitor from *P. gounellei* stem (PgTI)**

| Microorganisms | MIC (µg/mL)[a] | MBC/MFC (µg/mL)[b] |
|---|---|---|
| **Bacteria** | | |
| *Escherichia coli* | 37.5 | 75.0 |
| *Enterococcus faecalis* | 150.0 | ND |
| *Micrococcus luteus* | 37.5 | ND |
| *Pseudomonas aeruginosa* | 18.7 | ND |
| *Serratia* sp. | 7.5 | ND |
| *Staphylococcus aureus* | 7.5 | ND |
| *Staphylococcus saprophyticus* | 18.7 | ND |
| **Fungi** | | |
| *Candida albicans* | ND | ND |
| *Candida krusei* | 60.0 | ND |
| *Candida parapsilosis* | ND | ND |

*MIC: minimal inhibitory concentration, MBC: minimum bactericidal concentration, MFC: minimum fungicidal concentration, ND: not detected*

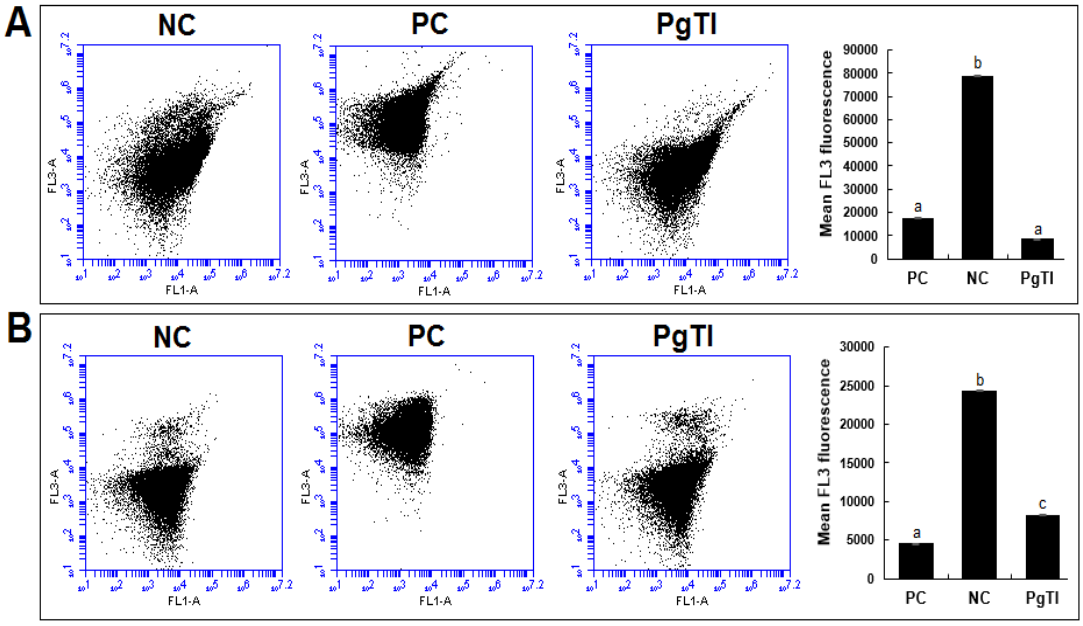


**Fig. 2. Flow cytometric analysis of the cell viability of *Escherichia coli* (A) and *Candida krusei* (B) treated with PgTI at the MIC. The negative control (NC) consisted of cells incubated in the absence of lectin. Isopropyl alcohol (70%, v/v) was used as a positive control (PC). FL1 vs FL3 dot plots are shown (counting beads are not shown)**

*The bar charts display mean fluorescence in the FL3 channel, which corresponds to the staining by propidium iodide. Data are expressed as the mean ± standard deviation (SD). Different letters indicate significant differences ($p < 0.05$) between treatments*

The viability of *E. coli* and *C. krusei* cells treated with PgTI at MIC was evaluated. PI staining (mean FL3 fluorescence) of *E. coli* treated with PgTI was not significantly different ($p > 0.05$) than in the negative control, indicating that cells were viable (Fig. 2A). For *C. krusei*, the mean FL3 fluorescence in PgTI-treated cells was significantly higher ($p < 0.05$) than in the negative control (Fig. 2B), indicating cell permeabilization due to membrane damage. The positive control had remarkably higher PI staining ($p < 0.05$) than untreated cells of both microorganisms.

The low MIC values detected in the antimicrobial assay suggest that the inhibitor is a good bacteriostatic and fungistatic agent against all susceptible species tested, except *E. faecalis*. The MBC/MIC ratio detected for PgTI against *E. coli* was 2.0, showing the inhibitor as a bactericidal agent [33]. Interestingly, PgTI did not damage the integrity of *E. coli* cells at the MIC, which shows that the bacteriostatic and bactericidal effects are well-differentiated regarding the inhibitor concentration. In certain situations, bacteriostatic agents can be preferable since the cell lysis caused by bactericidal agents may result in endotoxin surge and exacerbated inflammatory reaction [34].

A protease inhibitor from *Coccinia grandis* leaves showed antibacterial activity against *S. aureus* (MIC of 1.0 mg/mL), *B. subtillis* (MIC of 1.0 mg/mL) and *K. pneumoniae* (MIC of 100 µg/mL), values higher than those detected here for PgTI. This inhibitor also killed *E. coli* (MBC of 1.0 mg/mL; MIC of 0.63 mg/mL) and the authors suggested that it may have induced channel formations on the bacterial membrane resulting in out flowing of the cellular content [35].

PgTI inhibited growth of *C. krusei*, but the MFC value (neutralization of 99.9% of fungal cells) was not detected. Despite this, damage to the cell membrane was detected at the MIC. The inhibitor from *E. timbouva* seeds was an antifungal agent against *Candida albicans*, *Candida tropicalis,* and *Candida buinensis,* and also disturbed the integrity of the plasma membrane [21].

## 4. CONCLUSION

The stem of *P. gounellei* contains an anionic trypsin inhibitor stable to 50°C and active on medically important bacteria and the yeast *C. krusei*. Outcomes reported here reveal new insights into the biochemistry of *P. gounellei*, increasing the biotechnological value of this plant.

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## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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