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A Kunitz Proteinase Inhibitor from Corms of *Xanthosoma blandum* with Bactericidal Activity

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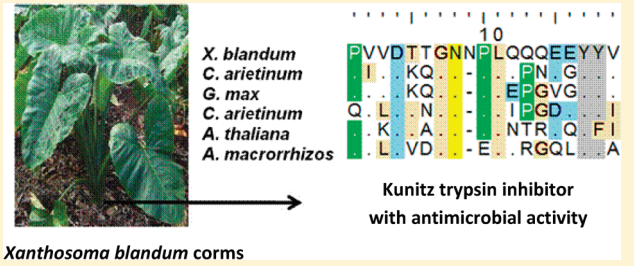
ABSTRACT: Bacterial infections directly affect the world's population, and this situation has been aggravated by indiscriminate use of antimicrobial agents, which can generate resistant microorganisms. In this report, an initial screening of proteins with antibacterial activity from corms of 15 species of the *Xanthosoma* genus was conducted. Since *Xanthosoma blandum* corms showed enhanced activity toward bacteria, a novel protein with bactericidal activity was isolated from this particular species. Edman degradation was used for protein N-termini determination; the primary structure showed similarities with Kunitz inhibitors, and this protein was named *Xb*-KTI. This protein was further challenged against serine proteinases from different sources, showing clear inhibitory activities. Otherwise, no hemolytic activity was observed for *Xb*-KTI. The results demonstrate the biotechnological potential of *Xb*-KTI, the first proteinase inhibitor with antimicrobial activity described in the *Xanthosoma* genus.



Nosocomial infections are a severe problem worldwide, particularly in poor and developing countries. These infections have been aggravated by antibiotic-resistant organisms, thus limiting treatment options.[1] As a result, urgent efforts are needed to find novel antimicrobials with different mechanisms of action in order to control bacterial infections.[2,3] To this end, several strategies have been developed to identify novel antimicrobials. The numerous compounds produced by plants are being screened by the pharmaceutical industry, which utilizes the molecules to produce drugs with greater efficacy and less toxicity. Latin America's traditional medicines include a large number of plants and phytoproducts known to possess potent medicinal properties, suggesting that phytoextracts may be useful for specific disease control. Among the various molecules with drug potential are proteinase inhibitors (PIs), which are capable of inhibiting the proteolytic activity of enzymes.[4] In general, they can interact with catalytic sites of serine-, aspartic-, cysteine-, and metallo-proteinases. These, in turn, determine the specificity and potential of inhibition.[5]

PIs are classified in a distinct class based on amino-acid sequences, localization of the reactive sites, disulfide bridge topology, mechanisms of action, three-dimensional structures, and stability.[6] A large number of inhibitors, especially serine proteinase inhibitors, have been isolated from an enormous variety of plants, including the Fabaceae, Solanaceae, and Poaceae families.[7] The Kunitz inhibitor type is one such serine proteinase inhibitor identified in plants. Kunitz-type serine proteinase inhibitors are polypeptides with molecular masses varying from 20 to 24 kDa, with four cysteine residues that form two disulfide bonds.[8] In general, they are present in seeds or tubers of plants, acting as storage protein. They exercise important functions in the development of seeds and sprouts, besides acting as a defense mechanism against predators and pathogens.[9−11] Members of the Kunitz family of inhibitors have major inhibiting activity against serine proteinases, mainly trypsin and chimotrypsin.[12] However, Kunitz inhibitors are also able to inhibit other proteinases, such as aspartic and cysteine proteinases.[13,14]

Kunitz inhibitors are stored in a number of plant tissues, including seeds,[15] tubers,[5,16,17] leaves,[18] rhizome,[19,20] fruits,[21] reproductive organs,[22] and leaves.[23,24] It is known that the process of subexpression and the location of these inhibitors in certain tissues depend on the genes that code for the production of these proteins; they may be expressed constitutively or as inducible plant defense genes.[21] According to Speransky and coworkers,[25] Kunitz-type protease inhibitors (KPIs) are induced during pathogen infection, suggesting that these inhibitors play a key role in defense against lytic enzymes involved in insect and pathogen attacks. In *Cicer arietinum*, screening of an epicotyl

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Table 1. Susceptibility of Human Pathogenic Bacteria to Protein-Rich Fractions[a] from Different *Xanthosoma* sp. Corms

	inhibition of growth (%)				
sample	*S. aureus*	*K. pneumonia*	*E. coli*	*P. mirabilis*	*S. typhimurium*
X. appendiculatum	–[d]	20 ± 2.6[b]	35 ± 4.1[b]	ND[c]	–[d]
X. blandum	15 ± 1.8[b]	–[d]	12 ± 0.8[b]	–[d]	25 ± 0.8[b]
X. brasiliense	–[d]	12 ± 0.9[b]	–[d]	–[d]	–[d]
X. mafaffa	–[d]	–[d]	7 ± 0.8[b]	–[d]	–[d]
X. maximiliani	–[d]	–[d]	–[d]	16 ± 3.1[b]	18 ± 4.2[b]
X. riedelianum	–[d]	ND[c]	21 ± 0.8[b]	ND[c]	–[d]
X. robustum	–[d]	ND[c]	ND[c]	ND[c]	15 ± 4.2[b]
X. saggittifolium	10 ± 1.8[b]	–[d]	50 ± 4.1[b]	17 ± 4.1[b]	–[d]
X. violaceum	–[d]	ND[c]	–[d]	ND[c]	11 ± 1.7[b]

[a] Samples were added at a final concentration of 100 $\mu g \cdot mL^{-1}$. PBS (pH 7.4) and chloramphenicol (40 $\mu g \cdot mL^{-1}$) were used as negative and positive controls, respectively. All analyses were performed in triplicate, and the activities were measured at OD 595 nm. [b] Values represent mean ± standard deviation of percentage reduction in optical density as compared to respective value of controls (100%). [c] ND, not determined. [d] –, no inhibition.

cDNA library revealed the presence of at least two different cDNAs encoding Kunitz-type inhibitors such as *CaTPI-1* and *CaTPI-2*, which express TPI-1 and TPI-2. The participation of TPI-2 in defense against mechanical damage[8,26] has been described. Speranskaya et al.[27] described the presence of four genes (PKPI-B1, PKPI-B2, PKPI-B9, and PKPI-B10) that express KPIs in *Solanum tuberosum* L. These also suppressed the growth and development of the phytopathogenic fungus *Fusarium culmorum*, corroborating the hypothesis that these inhibitors are expressed as a defense mechanism. In potato, they accumulate in leaves and tubers in response to mechanical wounding, UV radiation, and lesion by insects or phytopathogenic microorganisms.[28]

The most recent efforts to investigate Kunitz inhibitors have shown potential antimicrobial activity against fungi and Gram-positive and Gram-negative bacteria. Kim and co-workers[29] demonstrated that these inhibitors are synthesized in potato (*Solanum tuberosum* L.) tubers and that they strongly inhibited the growth of a wide variety of bacteria, including *Staphylococcus aureus*, *Listeria monocytogenes*, *Clavibacter michiganense*, and *Escherichia coli*, and fungi such as *Candida albicans* and *Rhizoctonia solani*. Furthermore, in seeds of *Acacia plumosa*, a Leguminosae-Mimosoideae plant, researchers have also isolated Kunitz inhibitors with antifungal activity.[30]

In view of the importance of inhibitors, the present study has been designed to determine the potential antibacterial activity of proteins in *Xanthosoma* genus corms. It also aims to evaluate their biotechnological potential for therapeutic drugs, in order to develop new strategies in the treatment of numerous diseases.

RESULTS AND DISCUSSION

Hospital environments have witnessed a progressive increase in resistance toward Gram-negative and Gram-positive bacteria. Thus, newly developed antimicrobial agents need to be evaluated for infection control. The antimicrobial potential of some plants from the *Xanthosoma* genus has been previously described. Kato and co-workers[31] demonstrated antibacterial activity of hydroperoxysterols from *X. robustum* against *Escherichia coli*, *Bacillus subtilis*, and *Micrococcus luteus*. Similar results have been reported by Schmourlo and co-workers,[32] in which *X. sagittifolium* extracts precipitated with EtOH showed higher activity against *Trichophyton rubrum*. These data led us to explore proteins and/or peptides from the *Xanthosoma* genus that could act as natural defenses against pests and pathogens. Thus, protein-rich fractions (PRFs) from different species of *Xanthosoma* were submitted to antimicrobial screening assays against different pathogenic bacteria (Table 1). Among these samples, the PRF from *X. blandum* revealed deleterious effects against *Staphylococcus aureus*, *Salmonella typhimurium*, and *E. coli*, showing 15%, 25%, and 12% inhibition, respectively, at a concentration of 100 $\mu g \cdot mL^{-1}$.

On the basis of recent studies and the potential activity of PRF from *X. blandum*, this sample was next applied to RP-HPLC, resulting in four major fractions: 7, 8, 9, and 10. These fractions were eluted with 49−70% acetonitrile dissolved in TFA 0.1% solution (Figure 1A). The data related to *Xb*-KTI purification are summarized in Table 2, showing that the three reported steps are sufficient for protein purification (20×) (Figure 1B). The antimicrobial activity of each fraction was evaluated *in vitro* against *S. typhimurium*, where fraction 7 showed clear inhibitory activity toward *S. typhimurium* development (Figure 2). Moreover, to improve the evaluation of inhibitory efficiency of *Xb*-KTI, the minimum inhibitory concentration (MIC) of *Xb*-KTI against this pathogenic bacterium was also calculated, showing a value of 10 μM (256 $\mu g \cdot mL^{-1}$) (Figure 2). This is a low MIC value when compared to other Kunitz inhibitors with antibacterial activity. For example PT-1, which caused deleterious effects against *Clavibacter michiganensis* development, showed an MIC of 50 μM (30 $\mu g \cdot mL^{-1}$).[28] In summary, *Xb*-KTI demonstrated more potent inhibitory activity against bacterial development, showing an MIC that was 5 times lower than that of PT-1.[28] These data suggest that this inhibitor could be used in the development of a therapeutic treatment against microorganisms and, due to the low concentration needed, may produce fewer toxic side effects.

A large number of proteins with antimicrobial activity were described in tubers and corms. Vivanco and co-workers[33] described two ribosome-inactivating proteins (RIPs) from roots of *Mirabilis expansa*, designated ME1 and ME, which showed activity against various bacteria, including *Pseudomonas syringae*, *Agrobacterium tumefaciens*, and *A. radiobacter*. In addition, ME1 and ME demonstrated activity against the fungi *Pythium irregulare*, *Fusarium oxysporum*, *Alternaria solani*, *Trichoderma reesei*, and *T. harzianum*. Flores and co-workers[34] showed that a protein named ocatin isolated from *Oxalis tuberosa* inhibits the growth of several phytopathogenic bacteria (*A. tumefaciens*, *Agrobacterium*

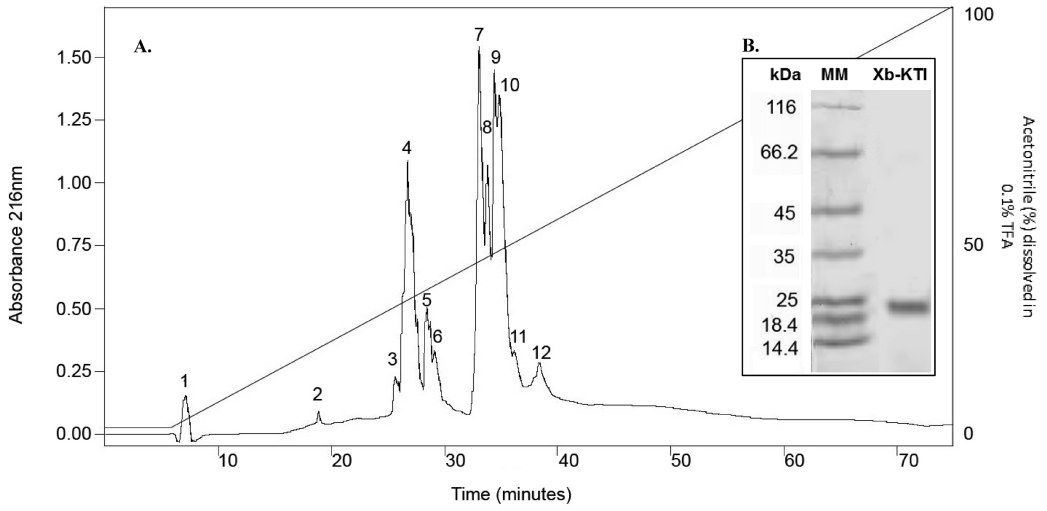

Figure 1. Chromatographic profile of *Xanthosoma blandum* proteins. (A) Protein-rich fraction was applied onto Vydac C-18TP reversed-phase chromatogram of retained proteins from corm kernels. (B) Tris-Tricine gel of RP-HPLC-eluted fraction of *X. blandum* proteins. MM indicates the molecular weight marker. The remaining lines correspond to RP-HPLC fractions. Black arrows indicate the sequenced protein. Diagonal line indicates nonlinear acetonitrile gradient (0−100%) dissolved in 0.1% TFA.

Table 2. Purification Steps of Kunitz Inhibitor from *X. blandum*

step	protein (mg)	total activity (UI)[a]	yield (%)	specific activity (UI·mg^{-1})	purification
crude extract	385.0	8400	100.0	21.7	1.0
rich fraction	249.7	56 000	64.9	225.0	10.4
Xb-KTI	21.2	9500	21.9	448.0	20.6

[a] One Kunitz inhibitor unit (UI) is defined as the inhibitor amount that decreased the absorbance at 595 nm by 0.01 OD in the antimicrobial assay conditions.

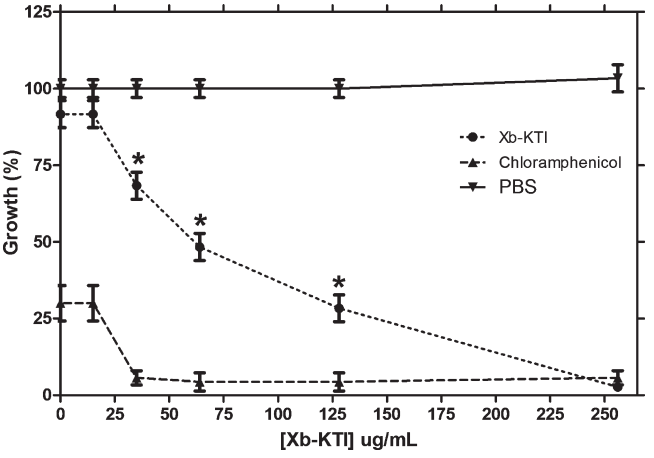

Figure 2. Evaluation of antibacterial activity against *Salmonella typhimurium*. Analyses were carried out using various concentrations of *Xb*-KTI (●), various concentrations of chloramphenicol 40 μg·mL^{-1} (▲), and PBS (▼). Asterisks represent significant difference with $p < 0.05$ for *Xb*-KTI versus chloramphenicol. All values are displayed as means ± standard deviation.

radiobacter, *Serratia marcescens*, and *Pseudomonas aureofaciens*) and fungi (*Phytophthora cinnamomi*, *F. oxysporum*, *Rhizoctonia solani*, and *Nectria hematococcus*). Furthermore, as can be seen in

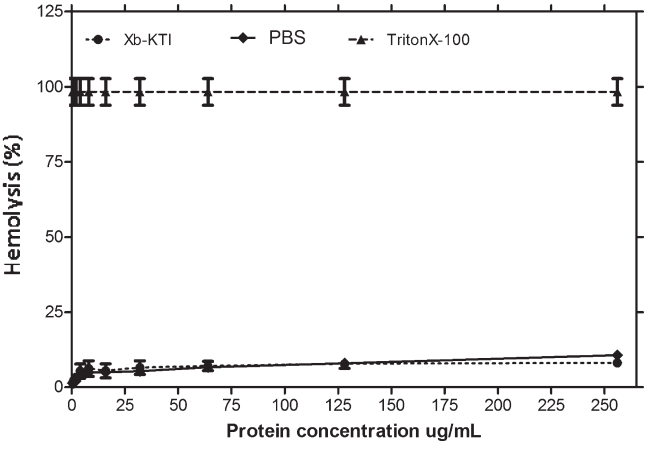

Figure 3. Hemolytic assay. A suspension of washed human erythrocytes was incubated with various concentrations of *Xb*-KTI (●). Values (OD540) were normalized against 100% lysed erythrocytes (0.1% Triton X-100) (▲) and PBS (♦) (untreated control). Asterisks represent significant difference with $p < 0.05$, *Xb*-KTI versus Triton X-100. All values are displayed as means ± standard deviation.

Figure 3, *Xb*-KTI showed no toxic effects toward mammalian cells, when human erythrocyte lyses are measured, according to what had been previously observed for the PT-1 proteinase inhibitor.

After its biological activity had been evaluated, fraction 7 was analyzed by Tris-Tricine gel, showing surprisingly a single band, with a molecular mass of about 24 kDa (Figure 1B). This protein band was submitted to Edman degradation, and its N-terminus was sequenced. Investigation of the 22 amino acid residues sequenced using FASTA3 showed different degrees of similarity to Kunitz inhibitor proteins with no evaluated antimicrobial activity (Table 3), suggesting that novel activities of those inhibitors should be found in the future. In general, Kunitz inhibitors are encoded in various parts of plants. Kunitz trypsin inhibitors were isolated from the seeds of *Inga laurina*,[35] *Tylosema esculentum*,[36] and *Cicer arietinum*,[37] with molecular masses of

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Table 3. Alignment of N-Terminal Sequence of *X. blandum Xb*-KTI with Other Kunitz Inhibitors from Several Plant Species

accession number	species	identity		function
this report	*X. blandun*		PVVDTTGNNPLQQQEEYYV	trypsin inhibitor and antibacterial activity
CAH61462	*C. arietinum*	63.16%	PIVDKQGN-PLQPNEGYYV	trypsin inhibitor
ACA23205	*G. max*	57.89%	PVVDKQGN-PLEPGVGYYV	putative trypsin inhibitor
CAB76906	*C. arietinum*	52.63%	QVLDTNGN-PLIPGDEYYI	trypsin inhibitor
NP_177373	*A. thaliana*	52.63%	PVKDTAGN-PLNTREQYFI	trypsin inhibitor
547741	*A. macrorrhizos*	47.37%	PVLDVDGNE-LQRGQLYYA	trypsin inhibitor
1922206B	*C. esculenta*	36.84%	PILDVDGNE-LRRGNRYYA	trypsin inhibitor

20 kDa. Roots from *Pseudostellaria heterophylla* also afforded one inhibitor with similar molecular mass.[38] Latex from *Carica papaya* presented an inhibitor of 24 kDa,[24] and *Solanum tuberosum* tubers gave inhibitors of 19 kDa[39] and 22 kDa,[40] among others. It is noteworthy that similarity in molecular mass also revealed a closer similarity (63%) with Kunitz-type inhibitors from *C. arietinum* (chickpea), belonging to the Fabaceae family.[8] Oliveira and co-workers[4] described the presence of various Kunitz inhibitors among other families, including Fabaceae, Solanaceae, and Araceae. The amino acid alignment analysis of the *X. blandum* N-terminus sequence with Kunitz inhibitors from other members of the Araceae family showed similarities of 47% with *Alocasia macrorrhiza* and 33% with *Colocasia esculenta,* suggesting that these inhibitors may be present in still more plants from the same family (Table 3). The present paper is the first report of this Kunitz inhibitor encoded in *X. blandum* and represents the first demonstration of its presence in the *Xanthosoma* genus.

In order to prove that *Xb*-KTI is a Kunitz inhibitor, this was challenged against bovine trypsin and also against serine proteinases from *S. typhimurium* (Figure 4). In response to this challenge, *Xb*-KTI caused 60% of inhibitory activity against bovine pancreatic serine proteinases and against almost 80% of proteinases extracted from *S. typhimurium* and previously evaluated in antimicrobial assays (Figure 4). Serine proteases have been commonly described as playing a role in the virulence factor of some bacterial strains, and a compound that acts to inhibit these enzymes could be used as an antimicrobial agent. Farn and Roberts[41] described the inactivation of HtrA caused by serine protease DegQ, where this effect caused an alteration in protease activities during infection because HtrA is an important virulence factor in *Salmonella enterica* serovar *Typhimurium*.[41] While recent studies identified another serine protease (virulence factor), designated Lon protease, this had less sensitivity than *E. coli* and *S. typhimurium* to UV light, other DNA-damaging agents, and cysteine protease inhibitors.[42]

Many antimicrobial proteins with associated protease inhibitor activity have been isolated from plants and characterized in detail. Among inhibitors from tubers, the existence of potamin-1 has been described. Named PT-1, this thermostable serine protease inhibitor of 5.6 kDa, which also has the ability to inhibit trypsin and chymotrypsin, showed antimicrobial activities against *Candida albicans*, *Rhizoctonia solani*, and *Clavibacter michiganense* exerted by PT-1.[28] After characterization of antimicrobial activity it was demonstrated that PT-1 presents hemolytic activity.[10] Moreover, potide-G, a peptide of 5578.9 Da, is capable of inhibiting growth of various bacteria, including *Staph. aureus*, *L. monocytogenes*, *C. michiganense*, and *E. coli*, and fungi such as *C. albicans* and *R. solani*.[29] Speranska et al.[27] also described Kunitz

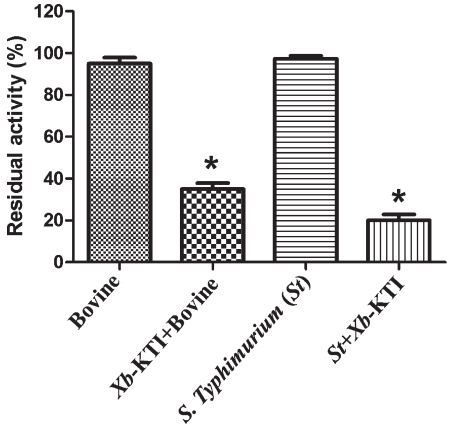

Figure 4. Inhibitory assays of *Xb*-KTI against bovine and *Salmonella typhimurium* serine proteinases (St). Asterisks represent significant difference with $p < 0.05$, bovine versus other treatments. All values are displayed as means ± standard deviation.

inhibitors from *S. tuberosum* with the ability to control *Fusarium culmorum* development. Similar Kunitz inhibitors with antifungal activity were also isolated from *Acacia plumose* seeds.[30]

However, it should be noted that inhibitors with antimicrobial activity are not exclusive to plants. The presence of PKPI has been demonstrated in the skin secretion of the tomato frog (*Dyscophus guineti*). This inhibitor was active against Gram-positive and Gram-negative bacterial strains and has antiviral activity against human herpes simplex virus and bovine parainfluenza virus.[43] In addition, it is also present in the hemolymph of the insect *Acalolepta luxuriosa*, where it can inhibit the growth of *M. luteus*, *B. subtilis*, *Bacillus cereus*, *Staph. aureus*, *E. coli*, *Aeromonas hydrophila*, *Erwinia persicinus*, and *S. marcescens* bacterial strains.[44] Thus, such inhibitors could play a vital role in protection against lytic enzymes involved in attacks by insects and pathogens, and they may become a biotechnological tool against human pathogens.

Data reported here add new information on the physiological role of corm proteins. The particular biological properties of the Kunitz inhibitor *Xb*-KTI against *Salmonella typhimurium* demonstrate its importance as a defense mechanism and the possibility of using these molecules as therapeutic drugs. In view of this potential new direction in disease treatment, it should be stressed that *Xb*-KTI is the first proteinase inhibitor to be isolated from the *Xanthosoma* genus. Other inhibitors with similar action are likely present in various plants, including others from the same genus. For these reasons, it is important to isolate and characterize these inhibitors and to evaluate the toxicological and pharmacological aspects of molecules obtained from plants. In terms

of biotechnology, this is a rich source that may provide many novel pharmaceutics and new directions in agricultural pest control.

A possible limitation for the use of *Xb*-KTI could be its large-scale production for medicinal purposes, which could be extremely expensive for large proteins with several disulfide bridges.[45] However, the production of recombinant antimicrobial proteins and/or peptides would be an alternative to reduce costs and avoid chemical synthesis. Another important issue that must be considered when Kunitz inhibitors are used to develop pharmaceuticals is the allergenic effect caused by this protein class.[46] Alternatives for the use of *Xb*-KTI and other inhibitors could involve the design of novel drugs by structural modifications or the use of only the region that is active toward microorganisms, which could reduce the side-effects of these compounds. In this first option, *Xb*-KTI could be used as a prototype compound. Moreover, patients could use Kunitz inhibitors as topical medication, as occurs with herpes simplex virus VP22 protein, used in the treatment of genital warts,[47] or as an agent for cleaning surgical equipment, acting in the prevention of infection.[48]

In summary, the results presented here add more knowledge about Kunitz inhibitors with antimicrobial activity. It is important to highlight that the protein discovered here will probably not be directly extracted from corms for use as a pharmaceutical agent. Instead, further studies to produce *Xb*-KTI as a commercial drug are necessary.

EXPERIMENTAL SECTION

Preparation and Preliminary Purification of Corm Extracts. Corms of the *Xanthosoma* genus were collected in a greenhouse at the Catholic University of Brasilia. These samples included *X. maximiliani*, *X. appendiculatum*, *X. sagittifoium*, *X. blandum*, *X. brasiliense*, *X. maffafa*, and *X. ridelianum*. These samples were macerated and extracted according to Costa and co-workers[47] with minor modifications. The corms were macerated with acetone, and the acetone was evaporated at room temperature. These samples were extracted with a solution containing 0.6 M NaCl and 0.1% HCl. The suspension was centrifuged at 4500*g* for 90 min, at 4 °C, and the supernatant was homogenized with Tris-HCl buffer at pH 7.5 for 3 h and precipitated with $(NH_4)_2SO_4$, at 25 °C. After centrifugation under the same conditions as described above, the precipitated material was resuspended in phosphate-buffered saline (PBS) 50 mM at pH 7.4 and dialyzed (3.0 kDa cutoff) against distilled H_2O. The extract was filtered and centrifuged at 4500*g* for 20 min at 4 °C, and the supernatant was separated and lyophilized. After determination of antimicrobial activity, 1 mg of the lyophilized fraction dissolved in 0.1% TFA was applied onto a reversed-phase HPLC (Vydac C-18 TP510 semipreparative column) equilibrated with 0.1% TFA. Proteins were eluted at a flow rate of 1.0 mL·min^{-1} with a linear MeCN gradient (0−100%) dissolved in 0.1% TFA solution and detected at 216 nm. For purification table construction, all steps were quantified by the Bradford method.[50] The values of optical density (OD) obtained from antimicrobial activity assays were transformed into inhibition units (IU). One unit was defined as the inhibitor amount necessary to decrease the absorbance of optical density by 0.01 in the antimicrobial assay previously described.

Molecular Mass Analyses. Molecular mass analyses and purity degree of HPLC protein fractions were determined by Tris/Tricine gel as described by Schagger and Von Jagow,[49] using 80 μg of proteins determined by fluorimetric assay (Qubit, Invitrogen). Bromophenol blue was used as tracking dye, and gels were silver stained.

Amino Acids Sequencing and *in Silico* Analyses. The N-terminal amino acid sequence of purified protein of 24 kDa was determined on a Shimadzu PPSQ-23A Automated Protein Sequencer performing Edman degradation.[50] PHT-amino acids were detected at 269 nm after separation on a reversed-phase C_{18} column (4.6 × 2.5 mm) under isocratic conditions, according to the manufacturer's instructions. Amino acid sequence was compared to SWISSPROT Data Bank, using the FASTA3 program.[51] Alignment of sequences was done by PBIL (Pole Bioinformatique Lyonnais) (http://pbil.univ-lyon1.fr/) using the ClustalW program[52] to analyze primary sequence similarities in each group.

Antimicrobial Bioassays. *Salmonella typhimurium* ATCC 14028, *Escherichia coli* ATCC 8739, *Klebsiella pneumoniae* ATCC13883, *Staphylococcus aureus* ATCC 29213, and *E. coli* ATCC 25922 were used for antimicrobial bioassays. The bacterial species were cultured in 1.0 mL of LB broth (10 g·L^{-1} NaCl, 5 g·L^{-1} yeast extract, and 45 g·L^{-1} bactopeptone) for 2 h, at 37 °C. Crude extract and protein-rich fraction were resuspended in 50 mM PBS (pH 7.4), filtered through 0.22 μm nylon membranes, and incubated at 512 and 100 μg·mL^{-1} final concentrations, respectively, with 5 × 10^6 cfu·mL^{-1} of each bacterial species for 4 h, at 37 °C. PBS (pH 7.4) and chloramphenicol (40 μg·mL^{-1}) were used as negative and positive controls, respectively. Bacterial growth was measured at 595 nm, every hour within the period of incubation, carried out according to protocols described by the National Committee for Clinical Laboratory Standards guidelines.[53] Each experiment was carried out in triplicate. In addition, to determine the MIC value, purified *Xb*-KTI was serially diluted from 2 to 256 μg·mL^{-1} in LB medium. In each well of a 96-well polypropylene plate, 100 μL of each dilution of *Xb*-KTI and 10 μL of cell suspension of bacteria were added (approximately 5 × 10^6 cfu of bacteria). The plates were incubated for 12 h at 37 °C. During this period the absorbance was measured in a plate reader (Bio-Rad 680) at 595 nm every 30 min.

Hemolytic Assays. Hemolytic assays used human erythrocytes as previously described by Kim and co-workers[28] with minor modifications. Erythrocytes were collected in the presence of the anticoagulant heparine and then were washed (×3) with 50 mM PBS (pH 7.4) and suspendend in the same buffer. The hemolytic activity of *Xb*-KTI was evaluated at standard concentrations ranging from 2 to 256 μg·mL^{-1} in association with aliquots of 25 μL of erythrocyte suspension. Erythrocytes were incubated with *Xb*-KTI for 30 min at 37 °C and then centrifuged for 5 min at 5000*g*. The hemolysis percentages were determined by measuring the supernatant optical density at 540 nm. PBS buffer (50 mM, pH 7.4) was used as negative control, and 0.2% Triton X-100 was used as positive control. Each assay was performed in triplicate.

Serine Proteinase Inhibitory Assay. The proteolytic inhibitory activity of *Xb*-KTI was evaluated using two methods: analyses to determine trypsin inhibitory activity and bacterial serine proteinase inhibitory assay. The average of trypsin inhibition was reached using 1% azocasein solution as substrate.[54] The trypsin inhibitory assay was performed by using 10 μL of trypsin at a standard concentration of 300 μg·mL^{-1} dissolved in 25 mM HCl solution and also 100 μL of purified inhibitor at a standard concentration of 22 μg.mL^{-1} dissolved in distilled H_2O. Enzyme concentration was obtained through the development of a curve-dependent concentration assay, where enzyme found on 1/2 V_{max} presented optical density between 0.25 and 0.30. The proteins were assayed against bovine trypsin (USB Corporation), performed in 50 mM Tris-HCl pH 7.5 buffer. The reaction for bovine trypsin was started with a 120 μL solution of 25 mM HCl, and after 30 min the reaction was stopped with 20% TCA. A 1% azocasein solution (200 μL) was used as substrate. The reaction was centrifuged at 12.000*g* for 10 min at room temperature, and 500 μL of supernatant was alkalinized by adding 500 μL of 2 N NaOH. The residual proteolytic activity was measured by absorbance at 440 nm. The blank control tests without the substrate were analyzed after 30 min. Reactions were quenched by the addition of 300 μL of a 20% TCA solution. All assays were conducted in triplicate. The evaluation of bacterial proteinase

inhibition was conducted with *S. typhimurium* ATCC 14028 cultured in 15 mL of LB medium (10 g·L^{-1} NaCl, 5 g·L^{-1} yeast extract, and 45 g·L^{-1} bactopeptone) overnight, at 37 °C. The bacteria were subcultured in 1 L of LB medium until the log phase established by previous curve growth, which corresponds to 2 h, at 37 °C and 240 rpm, according to protocols described by the National Committee for Clinical Laboratory Standards guidelines.[55] Bacterial growth was measured at 595 nm. The bacterial cells were all collected at the late exponential growth phase by centrifugation at 4000*g* for 15 min at 4 °C. The pellet was resuspended in extraction solution (HCl 0.1%, Tris 5 mM, $CaCl_2$ 5 mM, Triton X-100 0.1%, pH 7.5) and incubated in ice for 15 min. Freezing and thawing took place three times, followed by centrifugation at 4000*g* for 30 min at 4 °C. After this the supernatant was collected and the pool of protease from *S. typhimurium* with serine proteinase activity was assayed for evaluated inhibitory activity of *Xb*-KTI, according to the trypsin assay previously described.

Statistical Analysis. Data were analyzed by analysis of variance (ANOVA) and independent sample *t* test when the data showed normal distribution (GraphPad Prism, San Diego, CA). Results were considered to be significant at $p < 0.05$. All values are means ± SEM.

AUTHOR INFORMATION

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ABBREVIATIONS

ApTIA, *Acacia plumosa* serine protease inhibitors A; ApTIB, *Acacia plumosa* serine protease inhibitors B; ApTIC, *Acacia plumosa* serine protease inhibitors B; CaTPI-2, *Cicer arietinum* trypsin proteinase inhibitor 2; CaTPI-1, *Cicer arietinum* trypsin proteinase inhibitor 1; cfu, colony forming units; HPLC, high-performance liquid chromatography; KPIs, Kunitz-type protease inhibitors; LB, Luria−Bertani broth; MIC, minimum inhibitory concentration; PBIL, Pole BioInformatique Lyonnais; PIs, proteinase inhibitors; PKPI-B1, Kunitz-type proteinase inhibitor group B-1; PKPI-B10, Kunitz-type proteinase inhibitor group B-10; PKPI-B2, Kunitz-type proteinase inhibitor group B-2; PKPI-B9, Kunitz-type proteinase inhibitor group B-9; PRF, protein-rich fractions; PT-1, potamin 1; PPI, papaya proteinase inhibitor; RIP, ribosome-inactivating proteins; TFA, trifluoroacetic acid; *Xb*-KTI, *Xanthosoma blandum* Kunitz-type inhibitor

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