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Cocculus hirsutus trypsin inhibitor (ChTI) as a biocontrol agent for post harvest bacterial disease in tomato

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Abstract

The widespread distribution of proteinase inhibitors (PIs) in various plant parts has made them the most abundant class of defensive proteins. PIs restrict microbial developmental processes by attenuating respective proteases, making them a highly promising area of research. In our current investigation, we evaluated the bactericidal potential of a serine proteinase inhibitor (SPI), specifically the *Cocculus hirsutus* trypsin inhibitor (ChTI). Recombinant ChTI was expressed, purified using a Sepharose-4B affinity column, and utilized in bioassays against both gram-negative and gram-positive bacteria. Pre-incubation of bacterial trypsin-like proteinase (TLP) with 400 TIU/mg of ChTI resulted in TLP inhibition rates of 64%, 61.7%, and 56.4%, with IC_{50} values of 1.67 µg/ml, 1.74 µg/ml, and 1.90 µg/ml for *Xanthomonas axonopodis*, *Escherichia* coli, and *Ralstonia solanacearum*, respectively. Further evaluation against bacterial pathogens *Leuconostoc citreum* and *Lactobacillius fructivorans*, associated with postharvest tomato loss, indicated ChTI's potential in extending tomato shelf life. ChTI-treated tomatoes remained viable for over 20 days compared to 11 days for control fruits. Bacterial populations on treated fruits were significantly reduced, with ChTI inhibiting up to 49% and 36% of TLP for *L. citreum* and *L. fructivorans*, respectively. The inhibition pattern of bacterial proteinase was further confirmed through in-gel activity assays and bacterial cell mortality assays conducted by incubating live cells with ChTI. The study confirmed ChTI's strong affinity for bacterial cell wall proteins, as demonstrated by affinity column purification, SDS-PAGE, and western blotting. Overall, the inhibitory properties of ChTI, demonstrated

through bacterial bioassays, indicate its significant antibacterial activity and potential for post-harvest preservation.
Keywords: SPI (Serine proteinase inhibitor), ChTI (*Cocculus hirsutus* trypsin inhibitor), Gram-positive bacteria, Gram-negative bacteria, TLP (Trypsin like proteinase), Post-harvest preservation

1. Introduction

Plants are known to synthesize and accumulate defence related proteins *viz.* lectins, chitinases, thionins zeamatin, glucanases and proteinase inhibitors (PIs) to overcome biotic stress. Proteinases are the enzymes involved in protein digestion which have pivotal role in the regulatory life cycle of various living systems. Out of seven families of proteinases (MEROPS Database; http://merops.sanger.ac.uk), plant acquire five classes of endoproteinase (Ako and Nip, 2006), in which four are proteolytic enzymes based on active amino acids in their "reaction centre" and classified as Serine, Cysteine, Aspartic and Metallo proteases (Mourao and Schwartz, 2013). Proteinases are regulated by PIs (Molehin *et al.*, 2012; Rudzińska *et al.*, 2021; Sanrattana *et al.*, 2021). PIs interact with their target proteinases, resulting in the formation of stable proteinase-PI complex in turn regulating the proteolytic activity of their target proteinases by a competitive inhibition (Rustgi *et al.*, 2018; Hellinger and Gruber, 2019). Serine proteinase inhibitors (SPIs), have been extensively researched and are found in a wide range of organisms, from basic prokaryotic (bacterial) to advanced eukaryotic (human) systems (Rawlings *et al.,* 2016). PIs have been reported as antibacterial agents and their biological activity has been attributed to inhibition of bacterial proteinases (Kim *et al.,* 2009).

Bacterial growth is regulated due to the inhibition of bacterial protease by both natural as well as recombinant serine proteinase inhibitors (SPIs) (Bacha *et al.,* 2017; Zhoua *et al.,* 2018). Considering the importance of PIs in medicine and in agriculture as biocontrol agents, potentiality of *Cocculus hirsutus* trypsin inhibitor (ChTI) for its bactericidal effect was evaluated (Bhattacharjee *et al.,* 2009).

This study reports the isolation, purification and bactericidal effect of recombinant ChTI expressed in *E. coli*, targeting plant pathogenic bacteria and pathogens associated with the post-harvest diseases of tomatoes.

2. Materials and Methods

2.1 Extraction and Purification of ChTI

The extraction and purification of recombinant ChTI followed the procedures outlined by Bhattacharjee *et al.* (2009). In brief, a single colony of *E. coli* BL-21-pChTI was isolated and transferred to Luria Bertani broth (LB) containing 0.01% ampicillin. The culture was then grown until the OD_{600} reached 0.4-0.6. Isopropyl ß-D-1-thiogalactopyranoside (IPTG) was added to induce the expression of ChTI, and the culture was further incubated for 3-4 hours at 37 °C. The cells were harvested by centrifugation at 6000 g for 10 minutes. The resulting pellets were washed with 0.05 mM Tris-Cl (pH 7.6) and suspended in $1/10^{th}$ volume of the same buffer.

Cell lysis was achieved by sonication to isolate the total soluble protein (TSP). The TSP was then subjected to thermal denaturation at 70°C for 10 minutes, followed by snap chilling for 30 minutes to obtain heat-stable protein (HSP). Trypsin inhibitory activity (TIA) was measured using the Kunitz casein digestion method (Kakade *et al.*, 1969) and expressed as trypsin inhibitory units (TIU). The Trypsin Inhibitor was purified using a Trypsin-Sepharose affinity column.

2.2 Preparation of Inoculum

The antimicrobial activity of ChTI was evaluated against *Escherichia coli, Xanthomonas axonopodis,* and *Ralstonia solanacerum*. *E. coli* was cultured in LB medium, *X. axonopodis* was cultured in LB medium with 0.5% NaCl, and *R.solanacerum* was cultured in triphenyl tetrazolium chloride (TZC) medium.

2.3 Bactericidal Activity

2.3.1 Broth Dilution Assay

The antimicrobial activity of ChTI was assessed using a broth dilution method with some modifications. Bacterial cultures were grown in their respective growth media until they reached a density of 1.5×10^5 cells/ml. Serial dilutions were prepared to achieve a final density of 1.5×10^1 cells/ml, and these dilutions were used for the bactericidal assay with the trypsin inhibitor. 100µl each of the serially diluted bacterial cells were treated with various concentrations of ChTI (ranging from 2-40 µg/ml). The mixture was incubated for 2 h at 37 °C. 10 µl of each mixture (containing diluted bacterial cells and ChTI) were plated on agar plates and incubated at 37°C for 18 hours.

2.3.2 Evan's Blue Assay:

Post plating, the remaining cell suspensions from the previously conducted broth dilution assay were subjected to further incubation with 0.1% Evans Blue dye (0.1 g in 100 ml ethanol) for an additional 1 hour at 37 °C. Excess dye was washed with 70% ethanol. The mortality rate of bacterial cells at different ChTI concentrations was observed by measuring the absorbance at 595 nm and expressed as a percentage (%) mortality. For the control in the above experiment, bacterial suspensions were incubated with distilled water.

2.3.3 Proteolytic Activity:

Bacterial pathogens were cultured until they reached the late log phase, after which the cultures were centrifuged to obtain cell sediments in centrifuge tubes. The cell mass was washed three times and suspended in potassium phosphate buffer (PPB) (pH 7.4). Total soluble proteins (TSPs) were extracted by lysing the bacterial cells using sonication. The TSPs were subsequently assayed for trypsin-like activity (TLA) using the Kunitz casein digestion method (Kunitz, 1947). One unit of trypsin activity (TA) is defined as the enzyme activity that results in a 0.001 OD increase at 280 nm under the specified experimental conditions.

2.3.4 Trypsin Inhibitory Activity (TIA) of ChTI

The inhibitory activity of ChTI on bacterial proteases specifically trypsin was calculated as the difference between proteolytic activity with and without inhibitor. Trypsin inhibitory activity was assayed by Kunitz casein digestion method (Kunitz, 1947). One unit of TIA is defined as the trypsin inhibitor's activity, which inhibits or reduces one unit of Trypsin Activity (TA) under the said experimental conditions. The quantitative measurement indicating how much of ChTI is needed to inhibit bacterial TA *in vitro* by 50% (IC_{50}) was recorded.

2.4 Evaluation of *Cocculus hirsutus* Trypsin Inhibitor (ChTI) against Post-harvest Losses in Tomato

Tomato fruits of the Rasi Shivam variety were obtained from the University of Agricultural Sciences, Bangalore. The collected fruits were divided into unripe (UR), half-ripened (HR), and ripened (R) categories. A petiole dip and surface spray of the fruits were conducted using 3000 TIU/mg of ChTI. Phosphate buffer saline was used as a control in the experiment.

2.4.1 Identification of Post-harvest Bacterial Pathogen

Bacterial cultures were isolated from the control set (UR, HR, and R) for post-harvest bacterial population by swabbing the surface of the tomato fruits with sterile cotton swabs. Microbiological analysis of the samples was conducted using Nutrient agar, and the plates were then incubated at 37°C for 24 - 48 h. Distinct microbial colonies were isolated and sub cultured to obtain pure cultures.

2.5 Identification of Bacteria

16s rDNA sequencing was performed to identify the bacteria. PCR was performed using the Hi-Media Taq polymerase (500 U), 50 mM $MgCl_2$ (500 U) and 10X buffer (500 U) and dNTPs (10 mM each). Universal 16Sr DNA (Forward: 5'AGAGTTTGATCMTGGCTCAG3' and Reverse: 5'CTGCTGCSYCCCGTAG3'). (Waldeisen *et al.*, 2011). The PCR amplifications were performed with an Applied Biosystems Veriti Thermal cycler using the following cycling parameters: Initiate denaturation at 95°C for three minutes, followed by 30 cycles of denaturation at 94°C for 75 seconds, annealing of primers at 57°C for 60 seconds, and elongation at 72°C for 90 seconds, followed by a final extension at 70°C for ten minutes. Nucleotide basic local alignment and search tool (BLAST) was used to align the obtained sequences.

2.6 Isolation of Proteins having Trypsin Like Activity

The process described involves the extraction and purification of ChTI from bacterial cells. The process outlining the extraction, purification, and determination of the activity of ChTI from bacterial cells are mentioned as follows: First, the bacterial cells were grown until they reached an optical density of 0.4 at OD_{600} after which the cells were harvested by centrifugation at 6000 rpm at room temperature. The cells were then suspended in 10 mM Tris-Cl (pH 7.4) and sonicated for 10 minutes, followed by centrifugation at 12000 rpm for 15 minutes. The obtained pellet was washed with 10 mM Tris pH 7.4 four times and then suspended in 5 ml of 2% sodium dodecyl sulfate (SDS) in 10 mM Tris pH 7.4. The suspended mixture was incubated for 5 minutes in a boiling water bath, then cooled to room temperature and centrifuged at 15000 rpm for 10 minutes. The supernatant was precipitated with 5X volume of ethanol and 2% acetic acid at 5°C and centrifuged at 10,000 rpm for 10 minutes and the resulting pellets were suspended in 10 mM Tris (pH 7.4) and stored at 5°C from which a 5 ml aliquot of the mixture was loaded onto a ChTI affinity column. Lastly, the samples were eluted with 1% SDS and TLA and TIA of ChTI from the eluted fraction were determined using the Kunitz casein digestion method.

2.6.1 Alcian Blue Staining of Purified Bacterial Cell Wall Protein:

Known amount of eluted fraction was loaded onto SDS-PAGE and visualized by improved Alcian blue and silver nitrate staining (Moller and Poulsen, 1995). The procedure briefly involved fixing the gel with a solution of 10% acetic acid and 25% ethanol (Solution I) three times for 5 minutes each, followed by staining the gel with 0.125% Alcian blue for 15 minutes. The gel was then washed three times with Solution I, increasing the wash duration to 5 minutes each time. Next, the gel was sensitized by adding 5% glutaraldehyde for 5 minutes, followed by another wash with Solution I. Subsequently, the gel was thoroughly rinsed with water to remove any unbound Alcian blue. The gel was then stained with 0.4% silver nitrate for 10 minutes, washed with water, and the protein bands were developed using 2.5% NaCO3 and 0.01% formaldehyde. Finally, the gel was fixed by adding 10% glacial acetic acid.

3. Results and Discussion

3.1 Extraction of ChTI from Recombinant *E. coli* containing *pChTI:*

E. coli containing p*ChTI* were harvested and subjected to sonication to get total soluble proteins (TSP) which was further denatured at 70 °C to get heat stable fraction (HSPs). The HSPs were loaded on to the Trypsin Sepharose affinity column to get a purified fraction of ChTI. Trypsin Sepharose affinity column is commonly used for the purification of recombinant serine proteinase inhibitors (Bhattacharjee *et al.*, 2009; Mohanraj *et al.*, 2018). Bound ChTI was eluted using 0.2 N HCl (Fig. 1) and the eluent was immediately adjusted to pH 7.6 by adding 0.2 N NaOH. This resulted in 24.25-fold purification of ChTI suggesting the retention of functional integrity of the inhibitor. Affinity purified ChTI (Fig. 1, Table 1) exhibited 39,047 TIU/mg protein compared to TSP (1610 TIU/mg) and HSP (2140.0 TIU/mg). The most active fraction emerged out of the affinity column was subjected to SDS-PAGE on 12% gel (Laemmli, 1970). Silver staining of the SDS-PAGE gel revealed ChTI to be a monomeric protein with an apparent molecular mass of 18kDa (Fig. 1b). The result suggests that the inhibitor protein purified belongs to the Kunitz type of SPI family, whose members are mostly monomeric or dimeric with a molecular mass ranging from 18 to 26 kDa (Macedo *et al.* 2003).

3.2 Bioassay of ChTI against Bacterial Pathogens:

Inactivation of hydrolase enzymes and depolarization of plasma membrane of the pathogens are some mechanisms through which PI acts against phytopathogens and insects to retard their growth and invasive capability (Arulpandi and Sangeetha, 2012). SPIs also target serine proteases of gram-negative bacteria secreted on bacterial cell surface through active site binding. Intracellular expression of SLPI (secretory leukocyte proteinase inhibitor) was found to cause growth arrest in *E. coli*, accompanied by reduced protein synthesis (Dautin, 2010; Jamal *et al.*, 2013). Our investigation suggests that ChTI inhibits *E. coli* serine proteinase which is confirmed by trypsin like activity (TLA) assay, and bacterial mortality assay (Evans Blue; fig. 2).

Table 1. Fold Purification of Recombinant ChTI

Level of purification	**Protein Concentration (µg/mL)**	**Specific activity (TIU/mg protein)**	**Fold purification**
TSP	870.00	1610.00	1.00
HSP	810.00	2140.00	1.27
Trypsin Sepharose affinity purified fraction	210.00	39,047.00	24.25

TIU: Trypsin Inhibitory Units

Bacterial TLA was assayed using casein as substrate. Non-transformed *E. coli* cells showed an activity of 0.136 ± 0.006 TLA/mg protein (Table.2). Further evaluation of ChTI against plant pathogenic bacteria which include *Ralstonia solanacearum,* causal agent of wilt in more than 200 plant species, including crops like tomato, potato and peanut (Mansfield *et al.*, 2012) and *Xanthomonas axonopodis* pv. *punicae* causing bacterial blight in pomegranate leading to substantial losses in productivity was carried out (Sharma *et al.*, 2017). The highest TLA (0.286 ± 0.007 TLA/mg protein) was found in *X. axonopodis pv. punicae* compared to *R. solanacearum* (0.117 ± 0.004 TLA/mg protein). Post incubation with ChTI, the TLA was

reduced to almost 50% *i.e.* 0.103 ± 0.005 TLA/mg protein and 0.051 ± 0.003 TLA/mg protein respectively (Table 2).

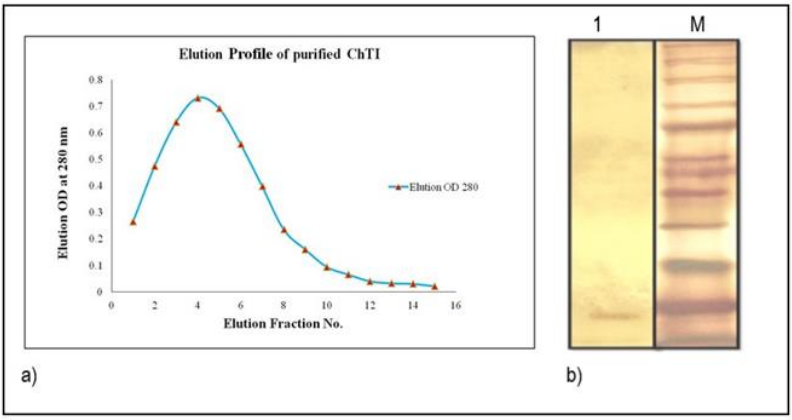

Figure 1. Purification of ChTI by affinity column chromatography
a) Elution Profile of purified ChTI, b) Visualization of ChTI by Silver Staining on 12% SDS-PAGE: Lane 1: 18kDa protein. M: Protein Marker (15-250 kDa)

Percent inhibition of bacterial TLA incubated with ChTI (400 TIU/mg protein) were found to be 64.0, 61.7 and 56.4 for *X. axonopodis, E. coli* and *R. solanacearum* respectively. LC_{50} of ChTI was found to be 1.67µg/mL, 1.74µg/mL and 1.90 µg/mL for *X. axonopodis, E. coli* and *R. solanacearum* respectively. Visible inverse relation with ChTI concentration with live bacterial cell mass (depicted as spots) on suitable agar medium were observed (Fig. 2) where increasing concentration of purified ChTI has shown to have inhibitory effect on each of the serially diluted log phase bacterial cells. Increasing concentration of ChTI from 16 µg/ml to 20 µg/ml shows increased cell mortality in case of *X. axonopodis* with 80% mortality at 20 µg/ml of ChTI. Undiluted cell mass of *E. coli* showed mild resistance to the increasing concentration of ChTI from 16 µg/ml to 18µg/ml whereas at 20 µg/ml of ChTI showed cent percent mortality. Similarly, *R. solanacearum* showed resistance to ChTI even at a concentration of 20 µg/ml but 40 µg/ml, ChTI showed cent percent mortality. Evan's blue dye binding to dead cells, indicates the cell mortality in a given system, the assay also suggested the same results (Fig. 2). The above results suggest that ChTI interacts with bacterial trypsin like proteins (TLPs). Trypsin like proteins is important part of bacterial cell growth, inhibiting which might have led to the growth arrest and mortality of bacterial cells (Jacyn Baker and Mock, 1994).

Table 2. Inhibitory activity of ChTI against gram-negative bacteria

Bacterial pathogens	**Caseinolytic activity (TLA) from bacterial pathogens**	**Residual TLA activity with 1000 TIU/ml of ChTI**	**% Inhibition of TLA**	**IC_{50} value of ChTI (TIU/ml tissue)**
	Mean ± SD			
Escherichia coli (DH5α strain)	0.136 ± 0.006	0.052 ± 0.003	61.7	910
Ralstonia solanacearum	0.117 ± 0.004	0. 051 ± 0.003	56.4	960

Xanthomonas axonopodis pv. punicae	0.286 ± 0.007	0.103 ± 0.005	64.0	830

TLA: Trypsin like activity, TIU: Trypsin Inhibitory Units

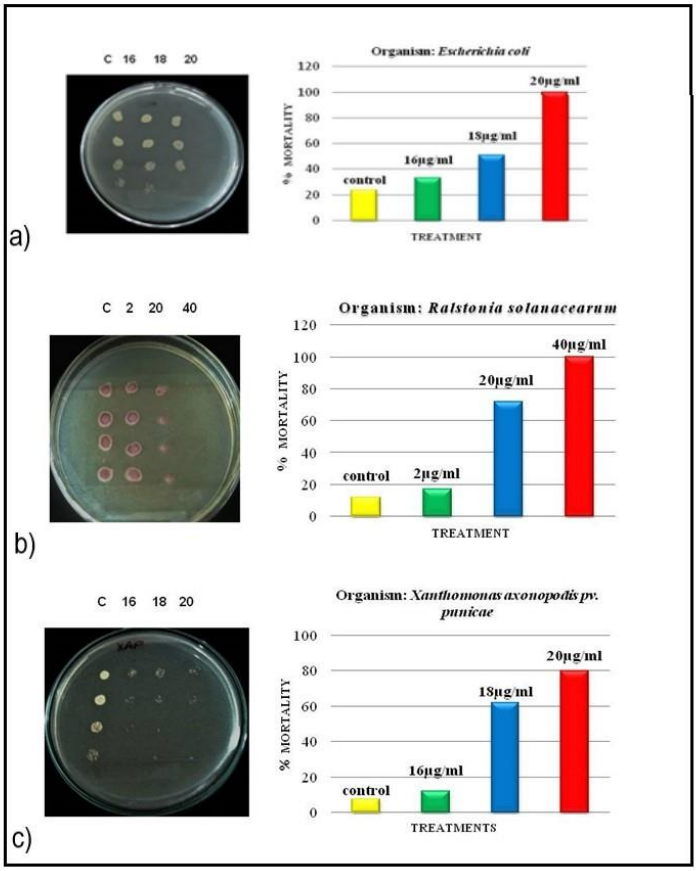

Figure 2: Evaluation of antibacterial activity of ChTI against different plant pathogens

a) Spot dilution assay of *E. coli* against different concentration of ChTI ranging from 16 – 20 µg. Bar Graph depicting % mortality of the *E. coli* against same concentration used in spot dilution assay.
b) Spot dilution assay of *R. solanacerum* against different concentration of ChTI ranging from 02- 40 µg/ml. Bar Graph depicting % mortality of *R. solanacerum* the against same concentration used in spot dilution assay.
c) Spot dilution assay of *X. axonopodis* against different concentration of ChTI ranging from 16-20 µg/ml. Bar Graph depicting % mortality of *X. axonopodis* against same concentration used in spot dilution assay.

3.3 Evaluation of ChTI against Postharvest Losses in Tomato

The control fruits in all three categories remained viable for 11 days, while ChTI-treated fruits remained viable for over 20 days (Fig. 3, 4). Surface swabs of the fruits were diluted to 10^{-3}, and the bacterial population at this dilution was studied. A comparison of the effect of ChTI on harvested tomatoes revealed a significant shelf-life enhancement of 9 days in ChTI-treated fruits. The high bacterial population in the control set and the decreased population in the ChTI-treated set indicate the effectiveness of ChTI as an effective surfactant (Fig. 5). The use of ChTI to control bacterial population for postharvest control is a novel approach.

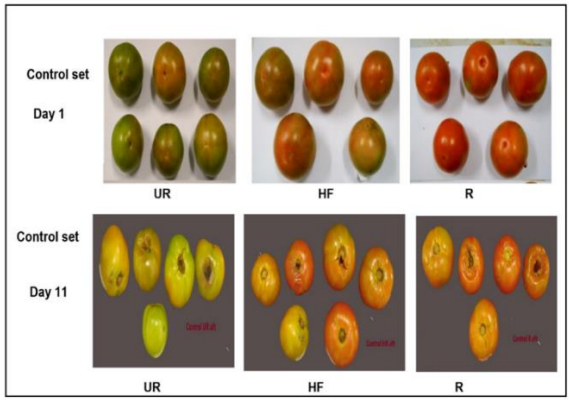

Figure 3. Tomato fruits of unripen (UR), half ripened (HR) and ripened (R) were treated using phosphate buffer as a control

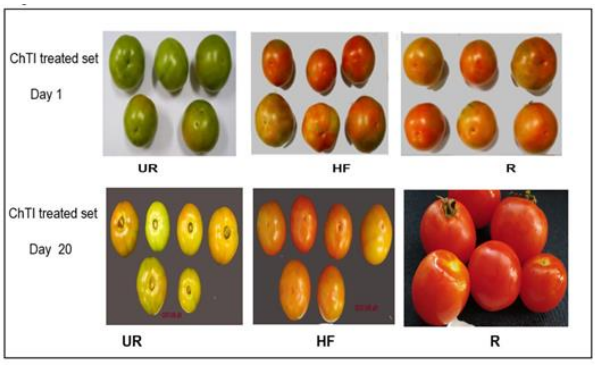

Figure 4. Tomato fruits of unripen (UR), half ripened (HR) and ripened (R) were treated using ChTI

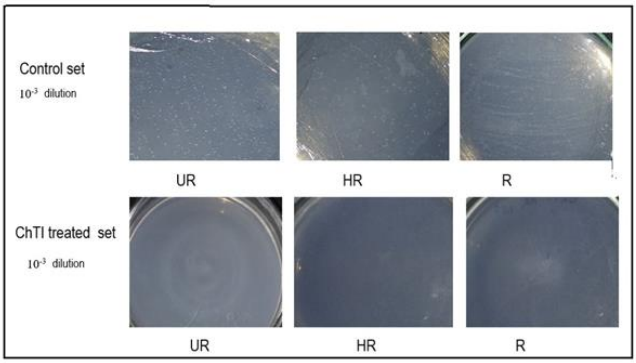

Figure 5. Fruit surface swabbed plates from different stages of harvesting unripen (UR), half Ripened (HR) and ripened (R) plated on Minimal media. Fever colonies in seen in ChTI treated fruit set in comparison to phosphate buffer treated set

3.4 Postharvest bacterial pathogen identification

Bacterial colonies were isolated from control tomato fruits using M9 minimal media, resulting in the observation of two distinct bacterial colonies. Subsequent sequencing and NCBI BLAST analysis identified the bacterium as *L. citreum* and *L. fructivorans*. (Figure 6). Post-harvest diseases of crops are commonly associated with fungal and bacterial pathogens. The most frequently isolated fungi include *Alternaria, Geotrichum*, Botrytis, Mucor, *Fusarium*, *Rhizopus*, *Colletotrichum* and *Penicillium*, while prevalent bacteria include *Acetobacter, Gluconobacter, Klebsiella, Leuconostoc, and Pectobacterium* (Ahmed *et al.*, 2017).

The production of cell wall degrading enzymes, oxalic acid, and the secretion of pathogenicity factors are important factors that can contribute to the virulence of different pathogens and their ability to cause disease in their hosts. Bacterial soft rot is a prevalent and potentially destructive postharvest disease caused by soft rot bacteria. The pathogen liquefies fruit tissue by breaking down the pectate "glue" that holds plant cells together, resulting in the liquefaction of fruit tissue. Bacterial soft rot caused mainly by four different bacteria. The primary soft rot bacteria, including strains of *Pectobacterium carotovorum*, *Pseudomonas*, *Xanthomonas*, and *Bacillus*, are responsible for causing soft rot in tomatoes. In addition, sour-rot diseases are caused by bacteria that produce lactic acid, such as Lactobacillus spp. and Leuconostoc spp. (Singh and Sharma, 2007).

Figure 6. a) PCR amplicons obtained using 16srDNA universal primers, lane 1- 500 bp PCR product for *L. fructivorans*, lane 2 – 1000 bp PCR product for *L. citreum*, and lane L -

100 bp DNA marker. NCBI BLAST results for the post-harvest bacterial pathogen identified as *L. fructivorans* and b, c) NCBI BLAST results for the post-harvest bacterial pathogen identified as *L. citreum*.

3.5 Determination of IC_{50} value of ChTI for postharvest bacterial trypsin-like activity

L. citreum and *L. fructivorans*, two organisms isolated and identified from control tomato fruits, were analyzed for caseinolytic activity. ChTI was found to inhibit up to 49% of *L. citreum* and 36% of *L. fructivorans* bacterial TLA, indicating its potential to inhibit the growth of post-harvest bacteria (Table 3).

3.6 Spot Dilution Assay of ChTI against *L. citreum* and *L. fructivorans*

The effectiveness of the ChTI inhibitor against *L. citreum* and *L. fructivorans* was analysed by spot dilution assay. There was a correlation with growth inhibition as the concentration of ChTI increased. At a concentration of 20 µg/ml, L. citreum exhibited a 50% reduction in growth, while L. fructovorans showed a 40% reduction. Complete inhibition was observed at 40 µg/ml. (Fig. 7; 1a, 2a). The Evans blue assay corroborated these findings, however, at 40 µg/ml, only 85% to 80% inhibition was noted. The observed deviation between the broth dilution assay and the Evans blue results may be attributed to differences in incubation durations, as the broth dilution assay was incubated overnight, whereas the Evans blue assay was incubated for only 2 hours. Additionally, the time required for the treatment to effectively act on bacterial cells is typically longer than 2 hours. Similar study on a serine proteinase inhibitor from the freshwater crayfish *Procambarus clarkia* demonstrated antibacterial activity against gram-negative bacteria such as *E. coli* and *K. pneumoniae*, as well as gram-positive bacteria including *B. subtilis*, *B. thuringiensis*, and *S. aureus* (Li *et al.*, 2009).

Table 3. Inhibitory activity of ChTI against gram-positive bacteria

Bacterial pathogens	Caseinolytic activity TLA from bacterial pathogens	Residual TLA activity with 1000 TIU/mL of ChTI	% Inhibition of TLA	IC_{50} value of ChTI (µg/ml)
	Mean ± SD			
L. citreum	79 ± 0.09	40 ±0.04	49	10.2
L. fructivorans	75 ± 0.029	48 ± 0.05	36	13.0

TLA: Trypsin like activity, TIU: Trypsin inhibitory units

The utilization of natural compounds like aloe gel and essential oils in the packaging process, alongside natural-based compounds, has proven to be effective in safeguarding packaged foods from microbial spoilage and extending their shelf life. This approach has surpassed the widespread use of chemicals and irradiation. (Montero-Prado *et al.*, 2011). In another study, Cherry tomatoes were coated with a formulation of hydroxypropyl methylcellulose and beeswax, combined with antifungal food additives. This coating effectively reduced Alternaria black spot and preserved the postharvest quality of the tomatoes during cold storage (Fagundes *et al.,* 2015).

3.7 Evans blue assay for validating percent mortality of postharvest bacterial cells

Evan's blue assay of *L. citreum* and *L. fructivorans* which specifically binds to the dead cells (Ohori *et al.*, 2007). The cells in the logarithmic growth phase were subjected to the Evan's

Blue binding assay method. The bacterial cells were treated with 10, 20, and 40 µg/ml of ChTI. The results showed a 45% mortality of *L. citreum* and a 35% mortality of *L. fructivorans* after treatment with 10 µg/ml of ChTI incubated for 1 hour (Fig. 7 1b, 2b).

3.8 Trypsin Inhibitor Sepharose Affinity Column Purification of Bacterial Cell Wall Protein

The trypsin inhibitor sepharose affinity column was used to investigate the interaction between bacterial cell wall protein and ChTI. This column was prepared by cross-linking purified ChTI, which had a concentration of 1200 TIU units per milligram of protein. Bacterial cell wall proteins from *L. citreum* and *L. fructovorans* were repeatedly passed through the column to ensure optimal binding. The bound proteins were then eluted using 2% SDS. The elution profile of the cell wall protein samples with the highest OD_{280} values was visualized on a 12% SDS-PAGE gel and stained using an improved Alcian Blue Silver stain method, which specifically targets bacterial cell wall-associated carbohydrates. The findings demonstrate a strong affinity of ChTI for the cell wall proteins of both *L. citreum* and *L. fructivorans* (Fig. 8a1-2, 8b1-2).

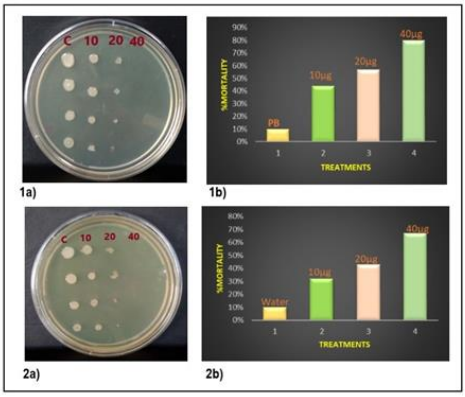

Figure 7. Evaluation of antibacterial activity of ChTI against different post-harvest pathogens

a) Spot dilution assay of *L. citreum* against different concentration of ChTI ranging from 10-40 µg/ml. b) Bar Graph depicting % mortality of the *L. citreum* against same concentration used in spot dilution assay.

b) Spot dilution assay of *L. fructovorans* against different concentration of ChTI ranging from 10 - 40 µg/ml. b) Bar Graph depicting % mortality of *L. fructovorans* the against same concentration used in spot dilution assay.

3.9 Western Blotting

The detection of ChTI-bound bacterial protein in Western blotting supports the findings from the alcian blue-stained SDS electrophoresis gel, confirming the binding of ChTI to the cell wall proteins of *L. citrovorum* and *L. fructivorans*. ChTI binds to the bacterial cell wall protein and inhibits the pathogens responsible for postharvest losses in tomato fruits (Fig.8 a1w, b1w). It has been established that ChTI binds to the mannosyl residues on the outer cell wall proteins, thereby inhibiting the bacterial pathogens *L. citreum* and *L. fructivorans.* Previous studies have reported that the mechanism of action of the Osmotin defence protein on *Saccharomyces cerevisiae* cell wall serine proteases, MNN2, MNN4, and MNN6, is inhibited by osmotin, leading to the inhibition of the organism's growth (Yun *et al.*, 1997). A similar mechanism of action is also observed in ChTI on yeast cells, wherein it binds to the outer cell

wall mannosyl residues with exposed mannosyl phosphodiester linkages, resulting in cell mortality (Bhattacharjee *et al.,* 2009).

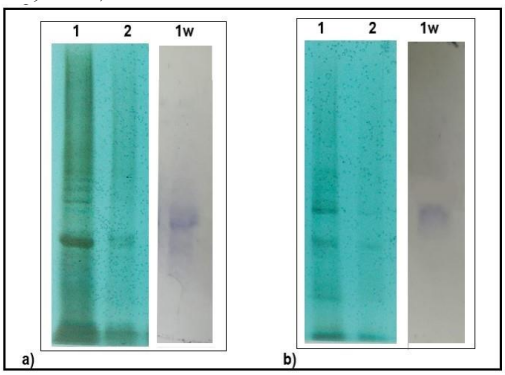

Figure 8. Visualisation of ChTI bound fractions by Alcian Blue staining and western blot
a) Lane 1: Alcian blue staining of crude *L.citrovorum* cell wall protein, Lane 2: Alcian blue staining of affinity column eluted *L.citrovorum* cell wall protein
Lane 1w- Cell wall protein of *L.citrovorum* detected by western blotting.
b) Lane 1: Alcian blue staining of crude *L. fructivorans* cell wall protein,
Lane 2: Alcian blue staining of affinity column eluted *L. fructivorans* cell wall protein
Lane 1w- Cell wall protein of *L. fructivorans* detected by western blotting.

4. Conclusions

In conclusion, our study brings forth tremendous bactericidal potential by this serine proteinase inhibitor from *Cocculus hirsutus*, which has been termed as ChTI. This inhibitor efficiently curtailed the growth of both gram-negative and gram-positive bacteria by attenuation of trypsin-like proteinases. Purified recombinant ChTI tested well in inhibiting the bacterial TLPs with high efficacy. IC_{50} values in the low-microgram-per-millilitre range have been shown for both gram-positive and gram-negative bacteria. Moreover, the shelf life of tomatoes extended due to a significant reduction of bacterial populations responsible for postharvest losses. The study confirmed that ChTI has high affinity to cell-wall proteins of bacteria, which makes it an effective antibacterial agent and hopefully a solution that could be used to extend the post-harvest life of products. The wide-spectrum activity of ChTI could be harnessed through the development of transgenic economically important crops to reduce the hazardous impact on the environment and make a substantial contribution to sustainable agriculture.

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Conflict of interests

The authors declare that they have no competing interests.

References

Ahmed, F. A., Sipes, B. S., & Alvarez, A. M. (2017). Postharvest diseases of tomato and natural products for disease management, *Afr. J. Agr.* Res., 12(9), 684-691.

Ako, H., & Nip, W. K. (2006). Enzyme classification and nomenclature. Food Biochemistry and Food Processing, 135-154, DOI:10.1002/9780470277577

Arulpandi, I., & Sangeetha, R. (2012). Antibacterial activity of fistulin: a protease inhibitor purified from the leaves of Cassia fistula. International Scholarly Research Notices, 2012(1), 584073, DOI:10.5402/2012/584073

Bacha, A. B., Jemel, I., Moubayed, N. M., & Abdelmalek, I. B. (2017). Purification and characterization of a newly serine protease inhibitor from Rhamnus frangula with potential for use as therapeutic drug. 3 Biotech, 7, 1-13, DOI: 10.1007/s13205-017-0764-z

Jacyn Baker, C., & Mock, N. M. (1994). An improved method for monitoring cell death in cell suspension and leaf disc assays using Evans blue. Plant Cell, Tissue and Organ Culture, 39, 7-12, DOI:10.1007/BF00037585

Bhattacharjee, C., Manjunath, N. H., & Prasad, D. T. (2009). Purification of a trypsin inhibitor from Cocculus hirsutus and identification of its biological activity. Journal of crop science and biotechnology, 12, 253-260, DOI: 10.1007/s12892-009-0094-8

Dautin, N. (2010). Serine protease autotransporters of enterobacteriaceae (SPATEs): biogenesis and function. Toxins, 2(6), 1179-1206, DOI:10.3390/toxins2061179

Fagundes, C., Palou, L., Monteiro, A. R., & Pérez-Gago, M. B. (2015). Hydroxypropyl methylcellulose-beeswax edible coatings formulated with antifungal food additives to reduce alternaria black spot and maintain postharvest quality of cold-stored cherry tomatoes. Scientia Horticulturae, 193, 249-257, DOI:10.1016/j.scienta.2015.07.027

Hellinger, R., & Gruber, C. W. (2019). Peptide-based protease inhibitors from plants. Drug discovery today, 24(9), 1877-1889, DOI:10.1016/j.drudis.2019.05.026

Jamal, F., Pandey, P. K., Singh, D., & Khan, M. Y. (2013). Serine protease inhibitors in plants: nature's arsenal crafted for insect predators. Phytochemistry Reviews, 12, 1-34, DOI:10.1007/s11101-012-9231-y

Kakade, M. L. (1969). An evaluation of natural vs. synthetic substrates for measuring the antitryptic activity of soybean samples. Cereal Chem., 46, 518-526.

Kim, J. Y., Park, S. C., Hwang, I., Cheong, H., Nah, J. W., Hahm, K. S., & Park, Y. (2009). Protease inhibitors from plants with antimicrobial activity. International journal of molecular sciences, 10(6), 2860-2872, DOI:10.3390/ijms10062860

Kunitz, M. (1947). Crystalline soybean trypsin inhibitor: II. General properties. The Journal of general physiology, 30(4), 291-310, DOI:10.1085/jgp.30.4.291

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. nature, 227(5259), 680-685, DOI:10.1038/227680a0

Li, X. C., Wang, X. W., Wang, Z.H., Zhao, X.F., Wang, J.X (2009). A three-domain Kazal-type serine proteinase inhibitor exhibiting domain inhibitory and bacteriostatic activities from freshwater crayfish Procambarus clarkii. Dev Comp Immunol. 33(12),1229-38, doi: 10.1016/j.dci.2009.07.001. Epub Jul 29. PMID: 19616577.

Macedo, M. L. R., Freire, M. D. G. M., Cabrini, E. C., Toyama, M. H., Novello, J. C., & Marangoni, S. (2003). A trypsin inhibitor from Peltophorum dubium seeds active against pest proteases and its effect on the survival of Anagasta kuehniella (Lepidoptera: Pyralidae). Biochimica et Biophysica Acta (BBA)-General Subjects, 1621(2), 170-182, DOI:10.1016/S0304-4165(03)00055-2

Moller, H. J., & Poulsen, J. H. (1995). Improved method for silver staining of glycoproteins in thin sodium dodecyl sulfate polyacrylamide gels. Analytical biochemistry, 226(2), 371-374, DOI:10.1006/abio.1995.1238

Molehin, A. J., Gobert, G. N., & McMANUS, D. P. (2012). Serine protease inhibitors of parasitic helminths. Parasitology, 139(6), 681-695, DOI:10.1017/S0031182011002435

Mohanraj, S. S., Tetali, S. D., Mallikarjuna, N., Dutta-Gupta, A., & Padmasree, K. (2018). Biochemical properties of a bacterially-expressed Bowman-Birk inhibitor from Rhynchosia sublobata (Schumach.) Meikle seeds and its activity against gut proteases of Achaea janata. Phytochemistry, 151, 78-90, DOI:10.1016/j.phytochem.2018.02.009

Montero-Prado, P., Rodriguez-Lafuente, A., & Nerin, C. (2011). Active label-based packaging to extend the shelf-life of "Calanda" peach fruit: Changes in fruit quality and enzymatic activity. Postharvest Biology and Technology, 60(3), 211-219, DOI:10.1016/j.postharvbio.2011.01.008

Mourão, C. B., & Schwartz, E. F. (2013). Protease inhibitors from marine venomous animals and their counterparts in terrestrial venomous animals. Marine drugs, 11(6), 2069-2112, DOI:10.3390/md11062069

Ohori, I. Y., Nagano, M., Muto, S., Uchimiya, H., & Kawai-Yamada, M. (2007). Cell death suppressor Arabidopsis bax inhibitor-1 is associated with calmodulin binding and ion homeostasis. Plant physiology, 143(2), 650-660, DOI:10.1104/pp.106.090878

Rawlings, N. D., Barrett, A. J., & Finn, R. (2016). Twenty years of the MEROPS database of proteolytic enzymes, their substrates and inhibitors. Nucleic acids research, 44(D1), D343-D350, DOI:10.1093/nar/gkv1118

Rudzińska, M., Daglioglu, C., Savvateeva, L. V., Kaci, F. N., Antoine, R., & Zamyatnin Jr, A. A. (2021). Current status and perspectives of protease inhibitors and their combination with nanosized drug delivery systems for targeted cancer therapy. Drug design, development and therapy, 9-20, DOI:10.2147/DDDT.S285852

Rustgi, S., Boex-Fontvieille, E., Reinbothe, C., von Wettstein, D., & Reinbothe, S. (2018). The complex world of plant protease inhibitors: Insights into a Kunitz-type cysteine protease inhibitor of Arabidopsis thaliana. Communicative & integrative biology, 11(1), e1368599, DOI:10.1080/19420889.2017.1368599

Sanrattana, W., Sefiane, T., Smits, S., van Kleef, N. D., Fens, M. H., Lenting, P. J., Maas, C. & de Maat, S. (2021). A reactive center loop–based prediction platform to enhance the design of therapeutic SERPINs. Proceedings of the National Academy of Sciences, 118(45), e2108458118, DOI:10.1073/pnas.2108458118

Singh, D., Sharma, R. R. (2007). Postharvest disease of fruit and vegetables and their management. In: Prasad, D. edition sustainable pest management. Daya Publishing House, New Delhi, India., pp. 183-189.

Sharma, J., Sharma, K. K., Kumar, A., Mondal, K. K., Thalor, S., Maity, A., Gharate, R., Chinchure, S., & Jadhav, V. T. (2017). Pomegranate bacterial blight: symptomatology and rapid inoculation technique for *Xanthomonas axonopodis* pv. punicae. Journal of Plant Pathology, 99(1), 109-119. http://www.jstor.org/stable/44280579.

Waldeisen, J.R., Wang, T., Mitra, D. and Lee, L.P. 2011. A real-time PCR antibiogram for drug-resistant sepsis. PLoS One 6(12): e28528.

Yun, D. J., Zhoa, Y., Pardo, J. M., Narasimhan, M. L., Damsz, B., Lee, H., Abad, L. R., Durzo, M. P., & Bressan, R. A. (1997). Stress proteins on the yeast cell surface determine resistance to osmotin, a plant antifungal protein. Proceedings of the National Academy of Sciences, 94(13), 7082-7087, DOI:10.1073/pnas.94.13.7082

Zhoua, C. Y., Liua, G., Chenga, X., Wanga, Q., Wanga, B., Zhanga, H., Hec, Q., & Zhanga, L. (2018). Antimicrobial activity of a newly identified Kazal-type serine proteinase inhibitor, CcKPI1, from the jellyfish Cyanea capillata. International journal of biological macromolecules, 107, 1945-1955, DOI:10.1016/j.ijbiomac.2017.10.069