**Xi'an Shiyou Daxue Xuebao (Ziran Kexue Ban)/**
**Journal of Xi'an Shiyou University, Natural Sciences Edition**
ISSN: 1673-064X
E-Publication: Online Open Access
Vol: 68 Issue 02 | 2025
DOI: 10.5281/zenodo.14842598

# GENE EXPRESSION, PURIFICATION, AND CYTOTOXIC ANALYSIS OF RECOMBINANT FUSION PROTEIN OF *CAJANUS CAJAN* TRYPSIN INHIBITOR WITH EPINECIDIN-1

**UNZILA YASIN**
Centre for Applied Molecular Biology, University of the Punjab, Lahore. *Corresponding Author

**ALEENA SUMRIN***
Centre for Applied Molecular Biology, University of the Punjab, Lahore.

**Abstract**

Trypsin inhibitors, also called serpins are the largest class of protease inhibitors having antagonistic properties to catalytic proteases. In this study, a 528bp *Cajanus cajan* trypsin inhibitor gene (CcTI) fused with a 63bp epinecidin-1 peptide (GFIFHIIKGLFHAGKMIHGLV), an anti-microbial peptide (AMP) from *Epinephelus coioides*, was cloned in pET-29a(+) expression vector and transformed in *E. coli* host system (*Top10* and *Bl21* cells) to produce rCcTI-epi-1 protein. Molecular cloning and chromatographic techniques were employed to maximize the production of biologically important trypsin inhibitory protein with a potent defensive role in therapeutics. The recombinant inhibitor-peptide complex (rCcTI-epi-1) was expressed in soluble lysate as well as inclusion bodies at reducing IPTG condition (0.5mM) with increasing the incubation time (24hrs). Contrary to insoluble protein aggregates, the expression levels of rCcTI-epi-1 protein were high in soluble lysate extracts. The 21kDa recombinant trypsin inhibitor-epinecidin-1 protein was purified by affinity chromatography at pH 7.5 with high purification yield but low purity of rCcTI-epi-1 protein using 250mM imidazole while elution with 500mM imidazole showed low yield but high on purity rCcTI-epi-1 protein. The rCcTI-epi-1 protein exhibited high microbial inhibition against *Escherichia coli* at a dose of 18µg/ml, whereas, the antimicrobial effect of rCcTI-epi-1 against *Staphylococcus aureus* was low (36µg/ml), mainly exerted by epinecidin-1 AMP peptide. The IC50 values of the cytotoxic effect of rCcTI-epi-1 protein against MDA-MB-231, MCF-7, A549, and HepG2 cell lines were 15.86µg/ml, 17.33µg/ml, 18.17µg/ml, and 20.25µg/ml, respectively. Taken together, our study investigate comprehensive invitro therapeutic potential of rCcTI-epi-1 protein against different carcinomas, uncovering the clinical potency of rCcTI-epi-1 as a targeted agent for cancer treatment in the future.

**Keywords:** Trypsin Inhibitor, *Cajanus Cajan*, Epinecidin-1, Expression Studies, Affinity Chromatography, Antibacterial, Cytotoxicity.

## INTRODUCTION

Molecular cloning and production of recombinant proteins are biological practices well-studied and routinely carried out in both prokaryotic and eukaryotic cells with *Escherichia coli* being the most preferred host organism, owing to its short generation time and quality protein expression. There are certain genes like trypsin inhibitor genes encoding for inhibitory proteins of proteases facing difficulty in translocations that are challenging for bacterial cloning (Vandierendonck, Girardin, De Bruyn, De Greve, & Loris, 2023). Trypsin inhibitors is a versatile group of proteins observed in all forms of life with a wide range of physiological applications in defense and therapeutics (Cisneros, Cotabarren, Parisi, Vasconcelos, & Obregón, 2020). These regulatory proteins are activated against

**Xi'an Shiyou Daxue Xuebao (Ziran Kexue Ban)/**
**Journal of Xi'an Shiyou University, Natural Sciences Edition**
ISSN: 1673-064X
E-Publication: Online Open Access
Vol: 68 Issue 02 | 2025
DOI: 10.5281/zenodo.14842598

pathogen attacks by establishing inhibitory canonical interaction with trypsin proteases found in the digestive tract of pests (do Amaral et al., 2022).

The proteolytic action of enzymatic inhibitors is the basis of their extensive use in the pharmaceutical industry. During the signaling of proteolytic cascades, trypsin inhibitors are responsible of reducing the incidences of tumorous growth, and improving diagnosis and treatments of cancers, cardiovascular diseases, genetic disorders, etc. (Cotabarren, Lufrano, Parisi, & Obregón, 2020). However, due to certain shortcomings, there are health conditions non-reactive upon administration of plant trypsin inhibitors as drugs with the synthetic class of inhibitors taking over their potential therapeutic function. The single-gene advantage of trypsin inhibitors renders them ideal candidates for creating recombinant inhibitor proteins (Habib, Zargar, & Fazili).

Naturally occurring trypsin inhibitors are rich in cysteine residues, crucial for maintaining structural stability by forming disulfide bridges. Inhibitors-trypsin protease complexes are held, regulated, and maintained by secondary bonds (disulfide bridges) (Laskowski & Laskowski Jr, 1954). Moreover, the expression of recombinant trypsin inhibitor proteins in *E. coli* host system is highly dependent upon the disulfide bonds for accurate translocation into the bacterial periplasmic space. Not all trypsin inhibitors are cloned and expressed solely using bacterial signal peptide, therefore, protein fusion tags are considered good alternatives for cellular translocations. The conjugation of peptides with trypsin inhibitors creates fusion protein benefitting the solubility, stability, and activity of the actual target protein (Malik, 2016).

The fusion concept is based on the small protein or peptide partners as tags adjoined with the help of linkers integrating and facilitating their native biological trait to their conjugated biomolecule. Fusion technology has been in practice for over the last 10 years with the aim to improve the actual effectiveness, biological potency, stability, and durability of different proteins in practical applications of agriculture, medicine, and research (Benchabane, Goulet, Dallaire, Côté, & Michaud, 2008).

Epinecidin-1 is a 21 amino acid cationic antimicrobial peptide with antibacterial, antifungal, anticancer, antiviral, and anti-immunomodulatory properties, etc. (Neshani, Zare, Akbari Eidgahi, Khaledi, & Ghazvini, 2019). Here, a *Cajanus cajan* trypsin inhibitor was fused with a short epinecidin-1 peptide to broaden the biological and therapeutic spectrum of the native biological trait of both independent proteinaceous entities. Trypsin inhibitors possess anti-proliferative potential inhibiting activity of the prognostic proteolytic enzymes, whereas, epinecidin-1 has bactericidal effects. CcTI gene when fused with an antimicrobial peptide, the conjugate develops cell-targeted properties offering protection against cancer and bacterial infections simultaneously, thus, promising profound curative outcomes by a multifunctional therapeutic agent (Jeyarajan et al., 2024).

In this study, the fusion concept was used for designing a fusion gene construct involving a *Cajanus cajan* trypsin inhibitor with epinecidin-1, a fish (*Epinephelus coioides*) peptide connected via a linker commercially synthesized in pET-29a(+) expression vector. The

**Xi'an Shiyou Daxue Xuebao (Ziran Kexue Ban)/**
**Journal of Xi'an Shiyou University, Natural Sciences Edition**
ISSN: 1673-064X
E-Publication: Online Open Access
Vol: 68 Issue 02 | 2025
DOI: 10.5281/zenodo.14842598

pET-29a/CcTI-epi-1 gene construct was cloned and expressed in *E. coli* host system followed by the enzyme kinetics study of the CcTI-epi-1 fusion protein against porcine trypsin. To confirm the biological potential, the recombinant trypsin inhibitor-epi-1 fusion protein expressed in *E. coli* was characterized for its bactericidal and cytotoxic properties.

## MATERIALS AND METHODS

### Structural analysis of CcTI gene

The physical parameters of the CcTI-epi-1 gene were determined using the Expasy ProtParam server (https://web.expasy.org/protparam/). The conserved domains and motifs in the CcTI-epi-1 gene were identified using InterProScan (http://www.ebi.ac.uk/interpro/search/sequence-search).

The CcTI-epi-1 sequence was subjected to identify the presence of signal peptide sequence using SignalP 4.1 server (https://services.healthtech.dtu.dk/services/SignalP-4.1/), a tool with about 78.1% accuracy rate in cleavage site prediction. COFACTOR server (https://zhanggroup.org/COFACTOR/) was used for structure-based functional annotation of the 3D structure model of CcTI-epi-1 gene predicting the gene ontology and enzyme classification (Khakha, Sharma, Kumari, Sahi, & Biswas, 2019).

**Table 1: FASTA sequences, amino acids, and accession no of fusion partners of CcTI-epi-1 protein**

| Gene | FASTA sequence | Amino acids | Accession no. |
|---|---|---|---|
| ***Cajanus cajan* trypsin inhibitor (CcTI)** | MVTDRDGDALRNGGTYHILPLFGVKDGGIEL ATTGNESCPLSVVQSPSGATFRGLPIRISSPY RVAYISEGLILSLAFASAPSCAPSPPKWTVVK GLPEGEAVKLPGYRSTVSGWFKIEKSSFEYL YKVVFCARGSDTCGDVGVSVDGGGVSRLVV TDDEGIFVEFMKGNSVDA | 191aa | NCBI accession no. >tr\|A0A151QR M2 |
| **Linker** | GGGGSGGGGSGGGGS | 15aa | {Samavarchi Tehrani, 2021} |
| **Epinecidin-1 peptide** | GFIFHIIKGLFHAGKMIHGLV | 21aa | (Chee et al., 2019) |

### Construction of CcTI-epinecidin-1 gene construct in pET-29a(+) expression vector

A 528bp *Cajanus cajan* trypsin inhibitor (CcTI) gene sequence retrieved with (accession no. >tr|A0A151QRM2) from NCBI database (https://www.ncbi.nlm.nih.gov/) was fused with a 63bp sequence of Epinecidin-1 (epi-1), an *Epinephelus coioides* peptide at the C-terminal region via a rigid G-S linker (Chee et al., 2019).

This gene-linker-peptide cassette was commercially synthesized in pET-29a expression vector within *Nde*I and *Xho*I MCS restriction sites from a supplier company Molecular Biology Products.

**Xi'an Shiyou Daxue Xuebao (Ziran Kexue Ban)/**
**Journal of Xi'an Shiyou University, Natural Sciences Edition**
ISSN: 1673-064X
E-Publication: Online Open Access
Vol: 68 Issue 02 | 2025
DOI: 10.5281/zenodo.14842598

### Molecular Cloning of pET-29a(+)-CcTI-epinecidin-1 expression vector in *E. coli* host system

The commercially developed lyophilized pET-29a(+)/CcTI-epi-1 vector was diluted (ng/µl) and transformed in *E. coli* Top 10 strain with 50µg ampicillin via $CaCl_2$ perforation followed by heat shock method. The transformed cells were plated on LB agar plates containing 50µg/ml of Kanamycin and incubated for 24 hours at 37°C. The above step was carried out for transformation in *E. coli* BL21 cells for protein expression. Clones were confirmed for positive transformants of the pET-29a(+)/CcTI-epi-1 gene by plasmid isolation, restriction digestion by *NdeI* and *XhoI* enzymes and colony PCR amplification.

### Protein expression via IPTG induction

Expression of the rCcTI-epi-1 protein was optimized using two concentrations of 0.5mM and 1mM IPTG inducer at a pre-induction cell density of OD 0.6. This IPTG-mediated optimization of protein expression was carried out in duplicates for induced and non-induced samples. Positively transformed Bl21 colonies were cultured in 15ml LB media overnight at 37°C. IPTG induction was given in the induced samples and incubated for 4-5 hours, followed by running samples on SDS PAGE prepared in 1X PBS and SDS loading dye. The 12.5% SDS analysis was done to assess the level of protein expression at different IPTG conc., compared to non-induced samples.

### Determination of CcTI-epi-1 protein in soluble and insoluble fractions

Shake flask incubation was carried out in 1litre LB media inoculated with CcTI-epi-1 gene transformed Bl21 colonies with 0.5mM IPTG induction at 37°C. Cells were centrifuged at 13,000 *x g* for 15mins and pallets were harvested in 100mM Tris-NaCl buffer (100mM Tris, NaCl, lysozyme) at room temperature for 1hr. The re-suspension was sonicated on ice with Misonix Ultrasonic Liquid Processors for 50sec at an amplitude of 20% for 5-6 cycles. The mixture was centrifuged at 13,000 *x g* separating the supernatant (soluble lysate) and pallet (insoluble protein aggregates). The pallet was re-suspended in the solubilization buffer (6M guanidine-HCL solution, NaCl) for 2 hours to release the protein content from the bacterial cells (Hong et al., 2020).

### Western Blot Analysis

The crude extract of rCcTI-epi-1 protein was transferred to the PVDF membrane. The membrane was blocked with TBS (containing 5% skimmed milk) and incubated overnight with monoclonal anti-His antibody (Sigma-Aldrich, St Lious, MOS, USA) at a dilution of 1:1000 at 4°C. The membrane was washed with TBS washing buffer 4 times followed by incubation with anti-His secondary antibody at a dilution of 1:5000 for 1hr at room temperature. The membrane was again washed with TB washing buffer and visualized after the addition of ρ-nitro blue tetrazolium chloride 5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) solution.

**Xi'an Shiyou Daxue Xuebao (Ziran Kexue Ban)/**
**Journal of Xi'an Shiyou University, Natural Sciences Edition**
ISSN: 1673-064X
E-Publication: Online Open Access
Vol: 68 Issue 02 | 2025
DOI: 10.5281/zenodo.14842598

### rCcTI-epi-1 protein purification by affinity chromatography

The rCcTI-epi-1 protein was purified through one-step affinity chromatography using affinity oriented spin column packed with pre-prepared 3ml of Ni-NTA charged affinity resin, the matrix. The affinity column was washed and pre-equilibrated with equilibration buffer pH 7.5 (20mM Tris, 300mM NaCl, 10mM Imidazole) thrice the volume of the resin bed. Additionally, the crude extract of rCcTI-epi-1 protein was further equilibrated with affinity resin at slight rotation for 1hr at room temperature. Protein binding was performed at a slow flow rate under a gravity flow system with equilibration buffer (10mM imidazole). The column was washed twice with a three-bed volume with washing buffer pH 7.5 (20mM Tris, 300mM NaCl, 25mM imidazole). Three fractions of rCcTI-epi1 protein were purified with 250mM and 500mM of elution buffer pH 7.5 (20mM Tris, 300mM NaCl, 250mM and 500mM imidazole). The purified rCcTI-epi-1 protein samples were analyzed on 12.5% SDS PAGE (do Amaral et al., 2022; Pirovani et al., 2010).

- **Enzyme kinetics of rCcTI-epi-1 protein**

### Residual enzymatic activity by trypsin inhibition assay

A 1ml mix of 0.5mg porcine trypsin solution, 0.5M sodium phosphate buffer, and 5ml of reagent grade water was prepared as the test sample, pH of solution was adjusted to 7.6 and incubated at room temperature for 30mins. An increasing concentration gradient ranging from 0.1uM-1uM of rCCTI-epi-1 protein was diluted with an aliquot of equal amount of water to determine the trypsin inhibition activity. Different concentrations of trypsin inhibitor was added and mixed in 1ml trypsin solution, inverted and incubated for 3-4mins at 25°C, achieving temperature equilibrium. The reaction began after the addition of 200ul of BApNA (N$\alpha$—benzoyl-D, L-arginine 4-nitroanilide hydrochloride) solution as a substrate. Absorption of trypsin/inhibitor solution was recorded at 410nm using spectrophotometer with control sample to compare the % inhibition potential of the rCcTI-epi-1 protein (do Amaral et al., 2022). The inhibition curve for determination of residual activity was calculated using formula:

$$\%\text{age residual activity} = \frac{\Delta\text{Absorption}_{410nm}\ \text{of Test}}{\Delta\text{Absorption}_{410nm}\ \text{of control}} \times 100$$

### Thermostability of rCcTI-epi-1 protein

Different aliquots of 0.5uM (500ug) of the rCCTI-epi-1 protein were incubated at six different temperatures of 40°C, 50°C, 60°C, 70°C, 80°C, and 90°C for 10min. In the inhibitor solution, 10ul of porcine trypsin (Sigma) at a concentration of 0.2mg/ml was added and incubated at 37°C for 15min. The reaction started after addition of 200ul of 1.2mM BApNA solution to the inhibitor/protease treatments. The substrate hydrolysis was carried and inhibition curve was made through the absorption of tested samples at 410nm against control without the inhibitor, stipulated as 100% inhibitory activity.

**Xi'an Shiyou Daxue Xuebao (Ziran Kexue Ban)/**
**Journal of Xi'an Shiyou University, Natural Sciences Edition**
ISSN: 1673-064X
E-Publication: Online Open Access
Vol: 68 Issue 02 | 2025
DOI: 10.5281/zenodo.14842598

### Antibacterial assay

The antibacterial potential of rCcTI-epi-1 protein was investigated by performing standard agar diffusion method against pathogenic bacterial strains of *Escherichia coli* and *Staphylococcus aureus*, procured from the Department of Microbiology and Molecular Genetics (MMG), University of the Punjab, Lahore, Pakistan. The bacterial cultures were inoculated in Luria-Bertani (LB) growth medium overnight at 37°C. The bacterial cultures were evenly distributed on L-agar plates using a three-dimensional swab technique. The plates were divided into five sections, each perforated with a hole. Amoxicillin was used as positive control and Tris buffer (pH 7.5) as negative control. The rCcTI-epi-1 protein was tested in triplicates i.e., 30µl (18µg/ml), 60µl (36µg/ml), and 90µl (72µg/ml) quantified by taking absorbance at 595nm. Plates were incubated for 24hrs at 37°C and inhibitory zones against *E. coli* and *S. aureus* were measured (mm) to observe the extent of microbial growth inhibition by rCcTI-epi-1 protein (Mehmood et al., 2020).

### Cell Cytotoxicity

Anti-proliferative activity of activated rCcTI-epi-1 protein was determined by performing MTT assay against four human cancer cell lines i.e. MDB-MB-231, MCF-7, A549 and HepG2 cancer cell lines obtained from CAMB and MMG cell culture laboratory, University of the Punjab, Lahore, Pakistan. Cells ($1 \times 10^5$) in their exponential growth were seeded in each well of 96 well microtiter plates and incubated in a $CO_2$ incubator at 37°C for 72hrs. Two-fold serial dilutions of rCcTI-epi-1 protein concentrations quantified by taking absorbance at 595nm were used in triplets with untreated cells as control and water as blank. Plates were incubated for 4 hours. Purple formazan crystals appear at bottom of wells. 100µl DMSO reagent was highly mixed with formazan crystals to form a single-cell suspension. Optical density was measured at 570nm using an ELISA plate reader to calculate the IC50 values and %age cell viability.

## RESULTS AND DISCUSSION

A potentially antitumor *Cajanus cajan* trypsin inhibitor (CcTI) protein and an antimicrobial epinecidin-1 (epi-1) peptide were used to create a fusion protein with dual biological properties. This synergism of a differentially active protein partners was designed to ensure a single streamlined fusion therapy that could help combat various health complications, minimizing off-target effects.

The structural analysis predicted presence of two motifs within the CcTI-epi-1 sequence confirmed by Motif Finder and InterPro servers. First motif spanning from 2 to 168 amino acid with protease inhibitory function from Pfam Kunitz legumes superfamily while the other belonged to Pleurocidin family (188 to 210 amino acids) with antimicrobial function (Figure_1a and 1b). A 26 amino acid N-terminal signal peptide sequence *D*-cutoff value of 0.782 against threshold value of 0.45 was identified in CcTI-epi-1 sequence by SignalP 4.1 as in Figure_2. Signal peptide was eventually removed from the designed fusion protein model as it does not have a role in mature protein.

**Xi'an Shiyou Daxue Xuebao (Ziran Kexue Ban)/**
**Journal of Xi'an Shiyou University, Natural Sciences Edition**
ISSN: 1673-064X
E-Publication: Online Open Access
Vol: 68 Issue 02 | 2025
DOI: 10.5281/zenodo.14842598

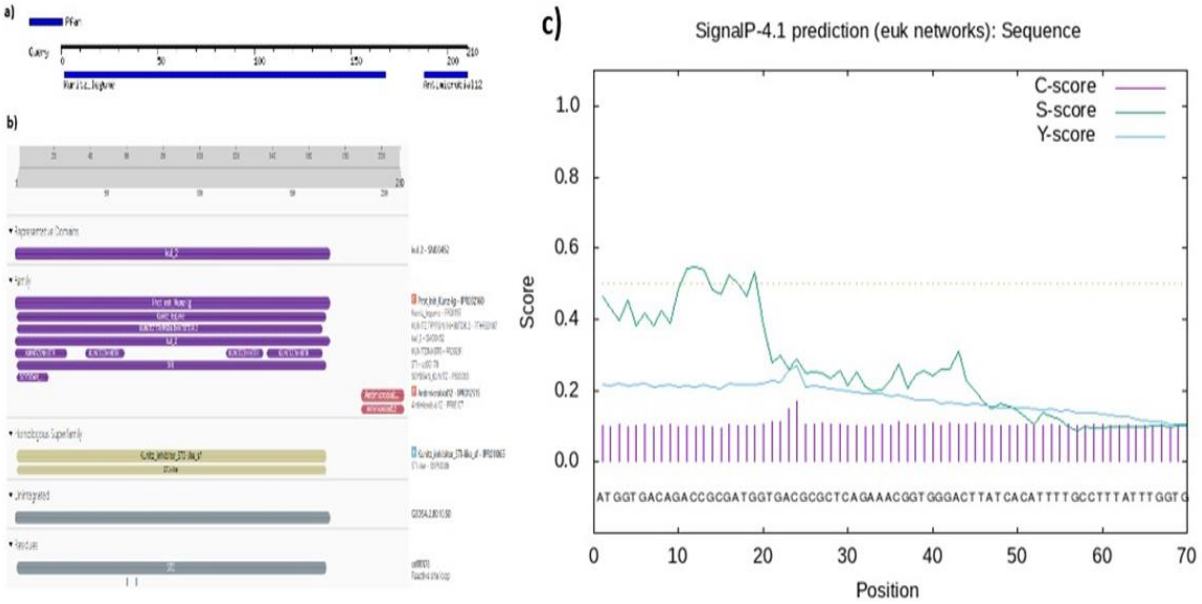
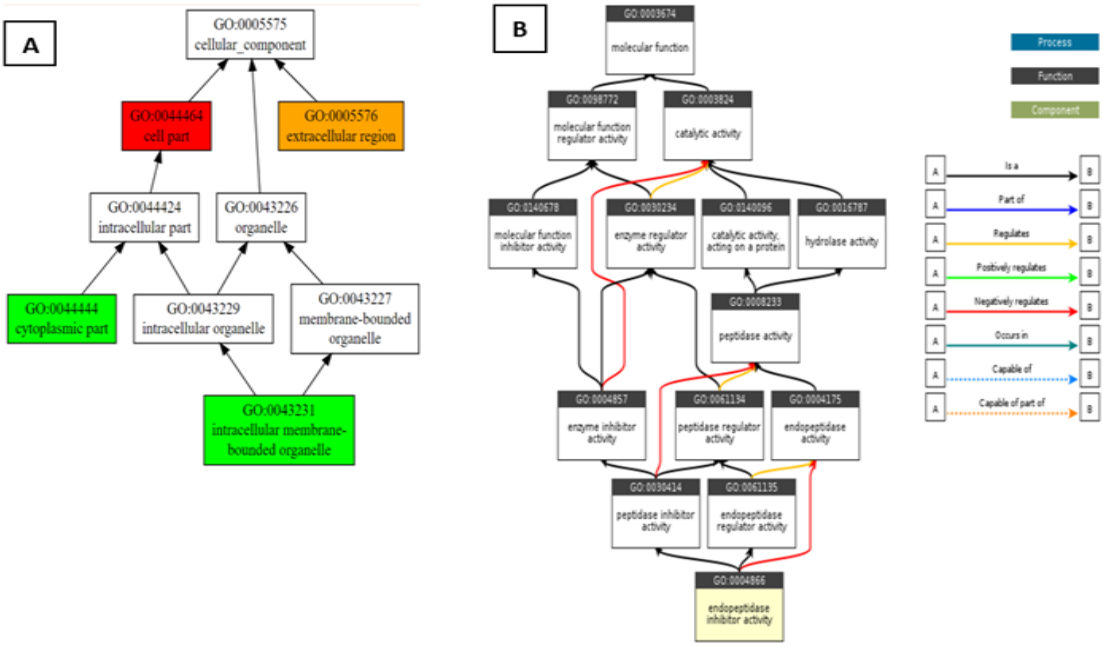



**Figure 1: a) Motif Finder and b) InterPro identified motifs in the CCTI-epi-1 gene. A specific portion (18-27 amino acid) was found to be homologous to the first ever identified soybean Kunitz trypsin inhibitor (shown in purple). c) Graphical representation the Signalp 4.1 server generated output peaks representing the presence of signal peptide at the N-terminal region of CCTI-epi-1 sequence**



**Xi'an Shiyou Daxue Xuebao (Ziran Kexue Ban)/**
**Journal of Xi'an Shiyou University, Natural Sciences Edition**
ISSN: 1673-064X
E-Publication: Online Open Access
Vol: 68 Issue 02 | 2025
DOI: 10.5281/zenodo.14842598

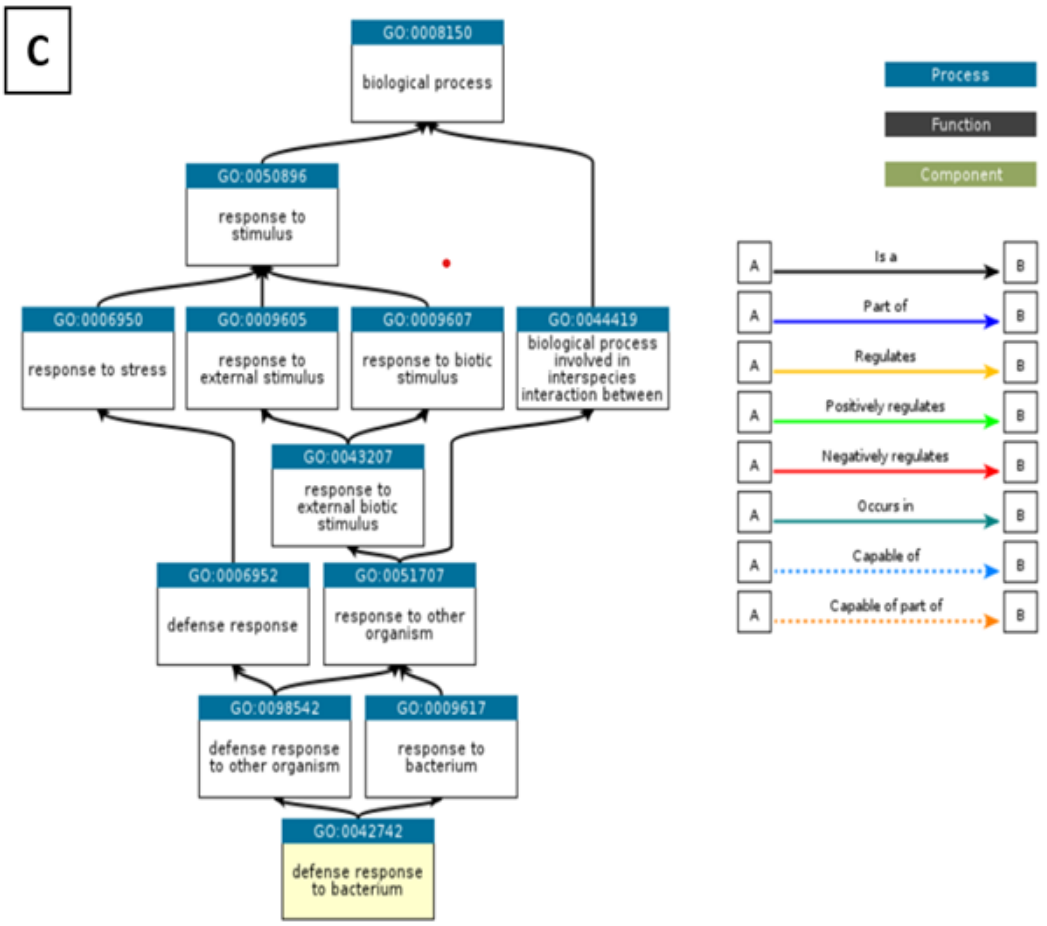



**Figure 2: COFACTOR predicted cellular location, molecular function, and biological function of the CCTI protein. (a) Cellular positioning of CCTI protein. Note: Red boxes, most probable designated protein function, yellow boxes are 2nd most preferred, green boxes is the least associated attribute of the CCTI protein as per the C-score. (b) Molecular function of CCTI protein. (c) Biological function of CCTI protein**

The functional annotation at molecular level for CcTI-epi-1 protein by COFACTOR server proposed gene ontology terms.

The GO term: 0044464 revealed protein to be expressed in cell part of the *E. coli* host system as cellular location with a C-score of 1.00 (Figure_2a).

Moreover, GO term: 0004866 defines the molecular endopeptidase inhibitor activity of protein with C-score of 0.97 (Figure_2b).

GO term: 0042742 predicts CcTI-epi-1 protein biological function as in defense responses to bacterium and GO term: 0050896 responsive to stimuli with a C-score of 1.00

**Xi'an Shiyou Daxue Xuebao (Ziran Kexue Ban)/**
**Journal of Xi'an Shiyou University, Natural Sciences Edition**
ISSN: 1673-064X
E-Publication: Online Open Access
Vol: 68 Issue 02 | 2025
DOI: 10.5281/zenodo.14842598

(Figure_2c). In 2019, a work plan by Khakha and peers on inter-alpha-trypsin inhibitor had role as serine endopeptidase inhibitor (Khakha, Sharma, Kumari, Sahi, & Biswas, 2019).

**Synthetic gene construction**

To construct a fusion CcTI-epi-1 protein, an N-terminal 528bp CcTI gene was fused with an active 21aa epinecidin-1 peptide at C-terminal with a $(G)_4S$ linker. The linker was used to ensure the cleavage of two fusion partners as fully functional proteins.

The final 636bp CcTI-epi-1 fusion gene sequence was synthetically introduced in pET-29a(+) expression vector (size-5371bp) within the MCS site of *NdeI* and *Xho1* enzymes.

The resultant 6007bp of pET-29a(+)/CcTI-epi-1 gene cassette (Figure_4) was confirmed for the accurate construct using Snap gene server and later commercially developed from supplier company “Molecular Biology Products”.

The purpose of designing a fusion construct was to create a single streamlined treatment that would address both cancer and bacterial infections simplifying multi-treatment regimens simultaneously. This introduction of a dual therapeutic approach was to clinically reduce the likelihood of resistance by treating different health problems by multiple angles.

**Nucleotide sequence of CcTI gene:**

**TGGTGACAGACCGCGATGGTGACGCGCTCAGAAACGGTGGGACTTATCACATTT**
**TGCCTTTATTTGGTGTGAAGGACGGCGGAATTGAACTTGCTACAACTGGTAATGA**
**GTCATGCCCCCTCTCTGTAGTTCAATCACCTTCAGGAGCCACCTTCAGAGGACTC**
**CCTATCCGTATCAGTAGTCCATACAGAGTGGCCTACATTTCGGAAGGCCTGATAC**
**TTTCGCTGGCTTTCGCGAGTGCCCCTTCATGTGCGCCTAGTCCACCCAAGTGGAC**
**TGTCGTCAAAGGACTTCCTGAGGGCGAGGCCGTTAAACTCCCTGGCTATCGTAG**
**CACAGTCTCAGGGTGGTTCAAAATTGAAAAGAGTTCGTTCGAATACTTGTATAAA**
**GTTGTCTTTTGTGCTCGGGGAAGTGACACCTGTGGCGACGTGGGAGTATCTGTAG**
**ATGGAGGAGGTGTGTCGAGACTTGTAGTAACCGATGATGAAGGAATCTTTGTCG**
**AATTTATGAAGGGAAACTCTGTCGATGCA**GGCGGCGGCGGATCCGGAGGAGGC
GGTTCAGGTGGTGGTGGATCA**GGCTTCATATTTCATATCATTAAGGGACTTTTCCA**
**TGCCGGCAAGATGATTCATGGCCTGGTG**

The pET-29a(+) vector consists of bacteriophage T7lac promoter promising a strong and tightly controlled gene expression. Initially, genes are cloned in a non-expressional E. coli host to maintain plasmid integrity due to absence of T7 RNA polymerase, keeping target genes of interest transcriptionally silent in un-induced

**Xi'an Shiyou Daxue Xuebao (Ziran Kexue Ban)/**
**Journal of Xi'an Shiyou University, Natural Sciences Edition**
ISSN: 1673-064X
E-Publication: Online Open Access
Vol: 68 Issue 02 | 2025
DOI: 10.5281/zenodo.14842598

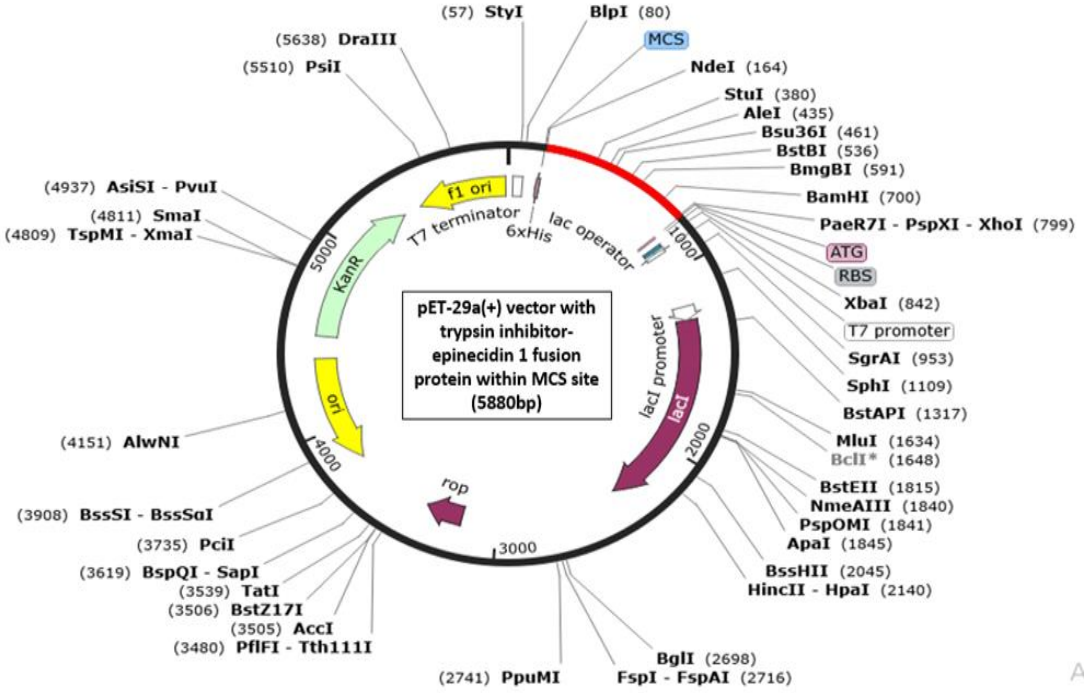



**Figure 3: Pictorial representation of pET-29a(+) carrying Cajanus cajan trypsin inhibitor fused with epinecidin-1 peptide (pET-29a(+)/CCTI-epinecidin 1)**

state. When established in a non-expression strain, plasmids are transformed in expression hosts like BL21 *E. coli* strain having a chromosomal copy of T7 RNA polymerase gene under control of *lacUV*5 promoter system, ensuring high level recombinant protein expression optimized by the addition of IPTG as an inducer (Microsoft Word - pET Manual 8th 0199pc.doc, 7/22/14).

## Cloning, Transformation, Gene amplification, and Restriction digestion

The synthesized pET-29a/CcTI-epi-1 vector was cloned in the *E. coli Top10* and *Bl21* competent cells (Figure_5a and 5b). The pET-29a(+)/CcTI-epi-1 gene transformed *Top10* clones were confirmed by double digestion using *NdeI* and

*XhoI* enzymes**,** generating a 636bp band of CcTI-epi-1 gene and a 6007bp band of pET-29a vector (Figure _5d). The open reading frame encoding of *Cajanus cajan* trypsin inhibitor protein (CcTI gene) was amplified using a set of primers: forward (F'-**CATATG**GTGACAGACCGCGATGGT) and reverse (R'-**CTCGAG**CATCGACAGAGTTTCCCTTCA) with *NdeI* and *XhoI* restriction sites at 3' and 5' end for identification of positive Bl21 transformants. The gene amplification was performed with ThermoScientific PCR master mix under the optimized PCR program (initial denaturation at 94°C for 5mins, followed by 35 cycles of denaturation at 95°C for 30sec, annealing at 58°C for 30sec, extension at 72°C for 1min, and final extension at 72°C for 10mins) in a 15µl PCR

**Xi'an Shiyou Daxue Xuebao (Ziran Kexue Ban)/**
**Journal of Xi'an Shiyou University, Natural Sciences Edition**
ISSN: 1673-064X
E-Publication: Online Open Access
Vol: 68 Issue 02 | 2025
DOI: 10.5281/zenodo.14842598

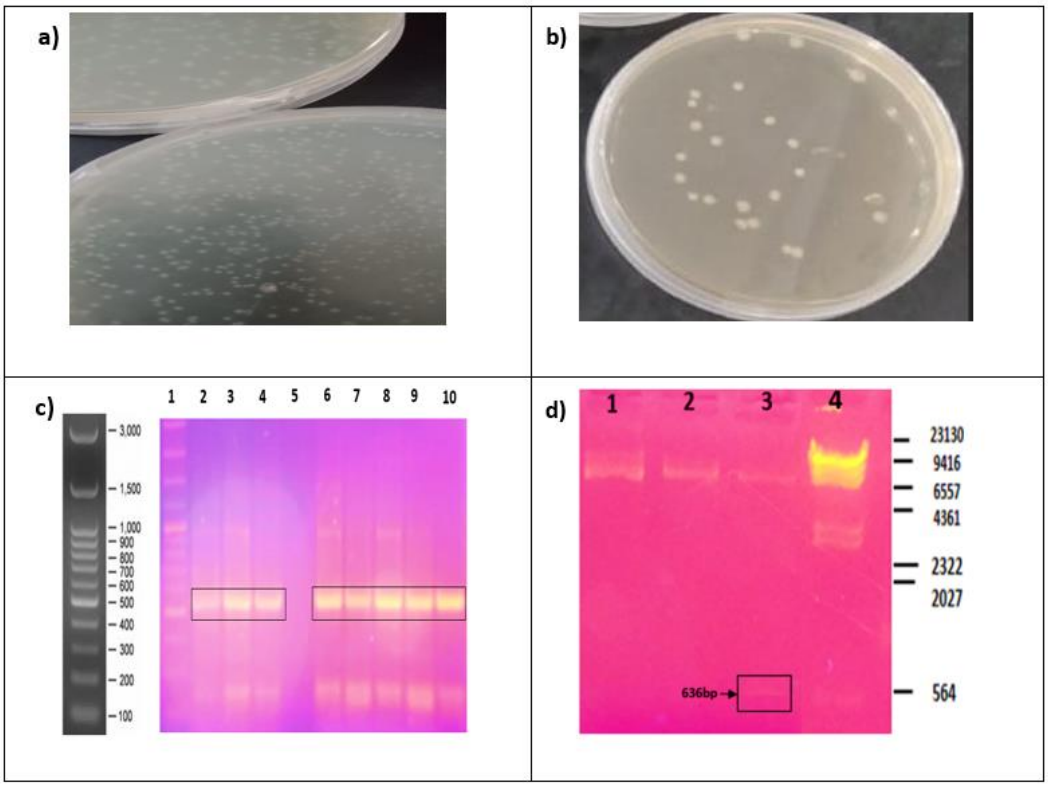



**Figure 4: Positive transformants of pEt-29(a)-CCTI-epi-1 (a) Top10 cloning and (b) Bl21 expression cloning. (c) Colony PCR amplification in 1% agarose gel electrophoresis, Lane-1, 100bp DNA ladder 'thermo: cat SM0323, Lane 2-9, positive transformants showing bright 528bp bands of CCTI gene, Lane-5, negative transformant with no cloned CCTI gene, (d) Restriction digestion in 1% agarose gel electrophoresis: Lane 1, a 5880bp plasmid isolated from Top10 positive clones carrying gene construct (pEt-29(a)/CCTI-epi-1), Lane 2, single digested by NdeI enzyme, Lane 3, double digested with XhoI enzyme resulting in a 636bp DNA fragment of CCTI-epi-1 gene digested from 5880bp plasmid showing positively transformed colonies, Lane-4, lamda DNA HindII 'thermo, cat no: SM0103**

reaction (0.9µl 2mM dNTPs, 1.5µl PCR buffer, 1.25µl $MgCl_2$, 1.5µl forward and reverse primers, 6.25µl PCR $H_2O$, and 2µl template) using thermocycler. A 528bp band of CcTI gene was amplified at 58°C (Figure_5c), exhibiting pET-29a(+)/CcTI-epi-1 gene transformed *BL21* colonies. The amplified product was quantified as ~400-500ng/µl via Nanodrop.

### Optimization of rCcTI-epi-1 protein expression under IPTG induction

IPTG is an inducer for protein expression that can hinder transcription if conc., is kept low or can cause cell cytotoxicity if conc. is high. The expression of recombinant proteins in

**Xi'an Shiyou Daxue Xuebao (Ziran Kexue Ban)/**
**Journal of Xi'an Shiyou University, Natural Sciences Edition**
ISSN: 1673-064X
E-Publication: Online Open Access
Vol: 68 Issue 02 | 2025
DOI: 10.5281/zenodo.14842598

pET-29a vector is controlled by T7 promoter induced by IPTG induction in the *E. coli* culture medium (Hong et al., 2020). The expression of induced and non-induced rCcTI-epi-1 protein samples was analyzed on 12.5% SDS Page showing a significantly elevated rCcTI-epi-1 protein expression at reducing 0.5mM IPTG conc., than at a high 1mM IPTG conc. (Figure_6a and 6b).

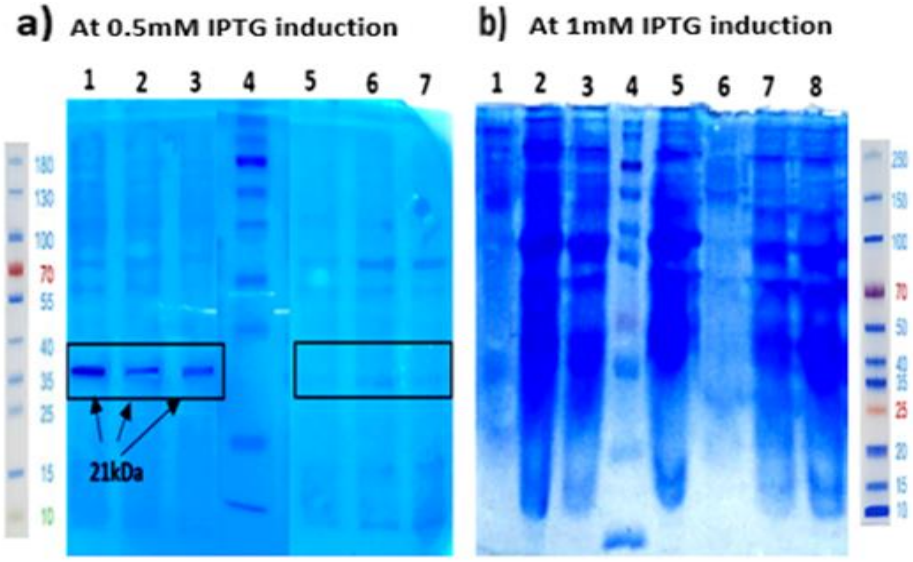



**Figure 5: 12.5% SDS PAGE analysis of induced fused gene construct of rCCTI-epi-1 protein at 0.5mM and 1mM IPTG concentrations. a) Lane 1-3, overexpressed 21kDa band rCCTI-epi-1 protein was obtained in the induced CCTI samples at 0.5mM IPTG conc., Lane 4, protein pre-stained marker 'thermo, cat: SM26616 Lane 5-7, very low expression in the non-induced rCCTI-epi-1 samples. b) Lane 1-3, At 1mM IPTG conc. no overexpressed 21kDa rCCTI-epi-1 protein band in induced samples, Lane-4, protein pre-stained marker Zokeyo, cat: 025A1, Lane 5-8, no overexpression of 21kDa rCCTI-epi-1 protein in non-induced samples**

In figure_6b, it was quite evident that an increase in IPTG conditions (1mM) resulted in loss of rCcTI-epi-1 protein expression, claiming 1mM IPTG induction a cytotoxic concentration for our targeted protein. The decrease in IPTG induction coupled with overnight induction incubation duration favored increased expression and water solubility of rCcTI-ep-1 fusion protein (Kwon, Chung, Yoo, & Chang, 2022).

**Determination of rCcTI-epi-1 protein in soluble and insoluble fractions**

Natural trypsin inhibitors also called serpins, are proteins produced in dual forms of soluble proteins as well as inclusion bodies. The cellular location of expression where protein would probably express in high concentration i.e. soluble (lysate) or insoluble fractions (pallet) is a crucial step to identify (Bhatt & Singh, 2020; Krishnan, Hedstrom, Hebert, Gierasch, & Gershenson, 2017; Pirovani et al., 2010). The 12.5% SDS PAGE (Figure 7) showed high level of rCcTI-epi-1 protein expression detected in both soluble

**Xi'an Shiyou Daxue Xuebao (Ziran Kexue Ban)/**
**Journal of Xi'an Shiyou University, Natural Sciences Edition**
ISSN: 1673-064X
E-Publication: Online Open Access
Vol: 68 Issue 02 | 2025
DOI: 10.5281/zenodo.14842598

and insoluble forms, however, a remarkably elevated expression of rCcTI-epi-1 protein at pH 7.5 was found in soluble lysate compared to insoluble fractions. This confirmed that rCcTI-epi-1 protein is highly expressed as soluble recombinant protein.

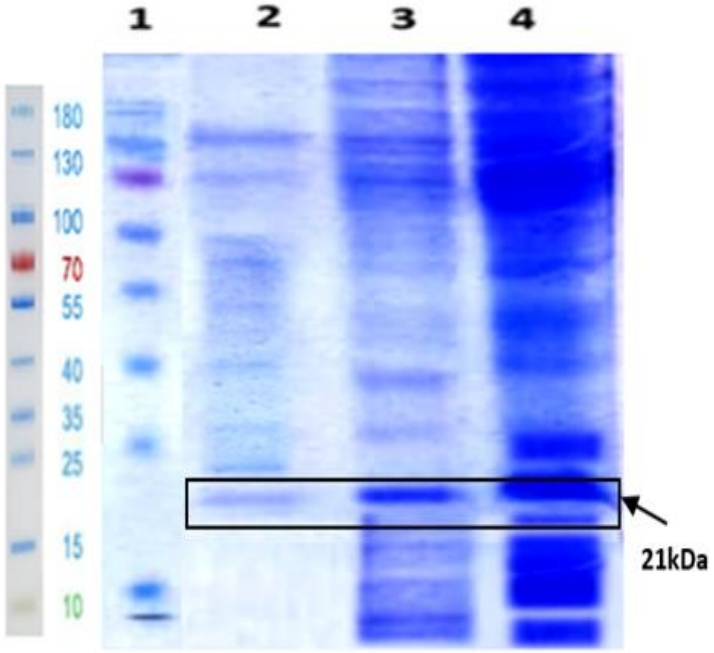



**Figure 6: 12.5% SDS PAGE gel analysis of the expression of 21kDa rCCTI-epi-1 protein in the soluble and insoluble fractions. Lane 1-2, expression of 21kDa rCCTI-epi-1 protein in the insoluble aggregates (inclusion bodies), Lane-3, Protein pre-stained marker 'thermo, cat: SM26616, Lane-4, high expression of 21kDa rCCTI-epi-1 protein in the soluble lysate compared to insoluble pallets**

A 2014 study on a rTI from *Psophocarpus tetragonolobus* purified from a soluble fraction of the bacterial cell lysate through affinity chromatography (Bhattacharjee, Banerjee, & Dutta, 2014). Such homologous results between our targeted recombinant *Cajanus cajan* trypsin inhibitor and recombinant *Psophocarpus tetragonolobus* trypsin inhibitor hints towards a close evolutionary relationship.

### Western Blotting

Using anti-His antibody, the protein of interest i.e. rCcTI-epi-1 protein in the total crude extract was identified. By means of western blot analysis, the anti-His antibody detected the presence of a single band with 21kDa protein density on the hybrid membrane, similar to the size our targeted rCcTI-epi-1 fusion protein. For control, empty Bl21 plasmid with no gene of interest was blotted on membrane, resulting in no protein detection by the antibody (Figure_8).

Xi'an Shiyou Daxue Xuebao (Ziran Kexue Ban)/
Journal of Xi'an Shiyou University, Natural Sciences Edition
ISSN: 1673-064X
E-Publication: Online Open Access
Vol: 68 Issue 02 | 2025
DOI: 10.5281/zenodo.14842598

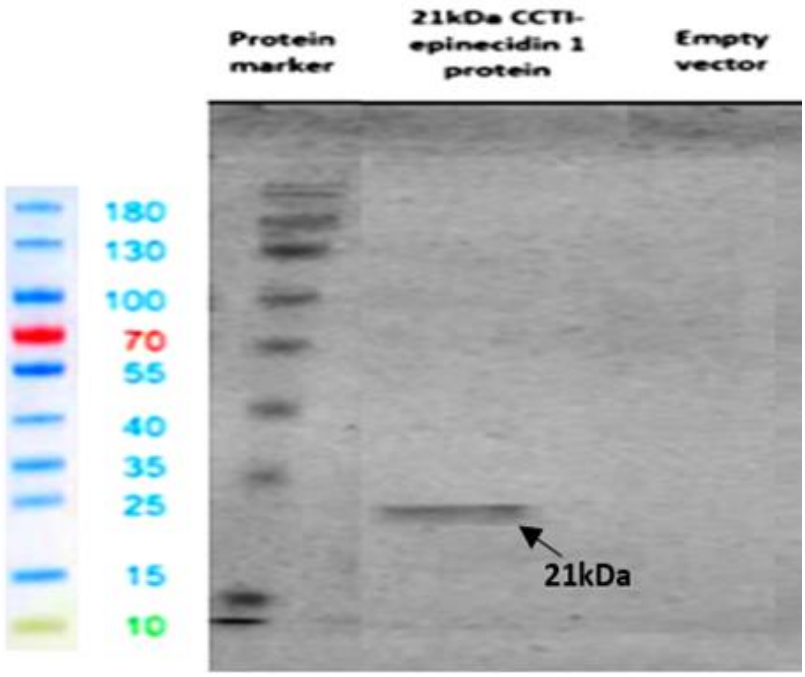



**Figure 7: Western Blot analysis of total protein extract of fused rCCTI-epinecidin 1 protein was analyzed on 12.5% SDS page. The anti-His antibody was used to immune-detect the presence of His-tag bearing recombinant protein. Lane-1: Protein pre-stained marker 'thermo, cat: SM26616, Lane-2: Crude protein extract containing 21kDa His-tagged rCCTI-epinecidin 1 protein, Lane-4: Empty Bl21 vector**

### Purification of rCCTI-epi-1 protein by affinity chromatography

The purification of suspension lysate (soluble fractions) containing rCcTI-epi-1 protein from the total crude extract was carried by affinity chromatography strategy using Ni-NTA affinity column. Binding of His-tagged rCcTI-epi-1 protein was mediated under 10mM imidazole conc. minimizing the binding of unwanted histidine rich proteins of bacterial host (Kielkopf, Bauer, & Urbatsch, 2020; Völzke et al., 2023).

During purification of rCcTI-epi-1 protein, a bright single 21kDa band of the protein was eluted using 250mM imidazole conc. at pH 7.5. Whereas, elution using 500mM imidazole resulted in lowered protein yield at pH 7.5 but with high purity (shown in Figure_9). The fractions with substantial protein yields were pooled for further protein functional assays. The purification protocol employed to obtain purified recombinant protein was satisfactory since the purified protein showed a single band on gel under reducing conditions. The procedure was safe ensuring no dimerization of the native protease inhibitor.

Most His-tagged protein purifies between 100mM and 250mM imidazole, however, elution is completely protein-dependent. Coupling the optimization of imidazole conc., for elution with pH chromatography buffers set according to protein isoelectric point (pI), are ideal

**Xi'an Shiyou Daxue Xuebao (Ziran Kexue Ban)/**
**Journal of Xi'an Shiyou University, Natural Sciences Edition**
ISSN: 1673-064X
E-Publication: Online Open Access
Vol: 68 Issue 02 | 2025
DOI: 10.5281/zenodo.14842598

conditions to purify his-tagged recombinant proteins by affinity chromatography (Kielkopf et al., 2020). Elution at 250mM imidazole conc., is stated to be an ideally optimized environment for purifying recombinant fusion proteins (Davis et al.).

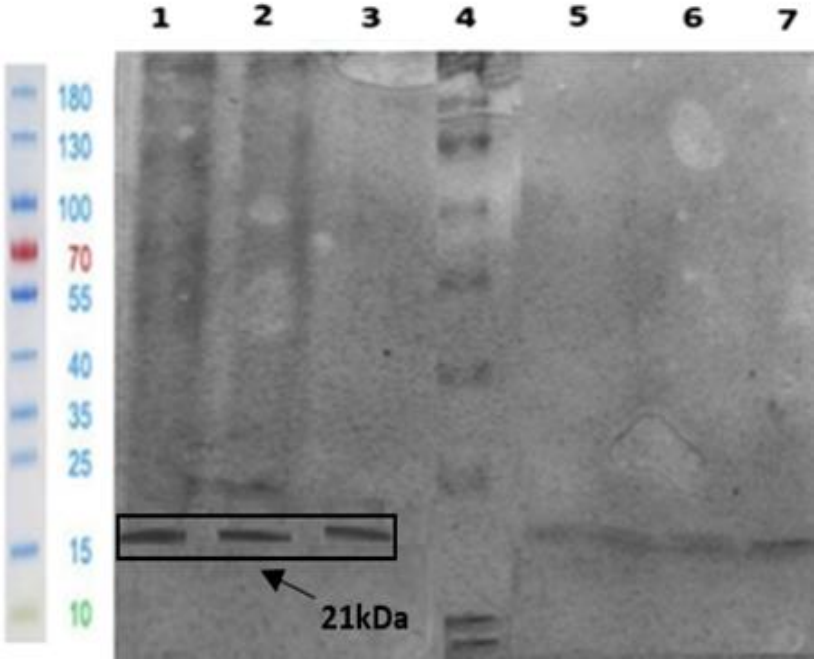



**Figure_8: SDS PAGE analysis of purified fused rCCTI-epinecidin 1 protein from Ni-NTA affinity chromatography. Lane 1-3, eluted fractions of bright 21kDa rCCTI-epi-1 protein band obtained using 250mM imidazole conc., Lane-4, Protein pre-stained marker 'thermo, cat: SM26616, Lane 5-7, eluted fractions with low yield of 21kDa band rCCTI-epi-1 fusion protein obtained using 500mM imidazole conc. during affinity-based purification**

### Residual enzymatic activity of rCcTI-epi-1 protein

The residual enzymatic activity of rCcTI-epi-1 fusion protein against porcine trypsin showed trypsin activity decreases linearly with an increasing gradient of inhibitor. The purified rCcTI-epi-1 protein produced was active to inhibit the trypsin activity in a linear fashion. With a treatment of 0.2uM of rCcTI-epi-1 protein, the trypsin activity was decreased to 87.04%, whereas, at a conc. 1uM of rCcTI-epi-1 protein there was a significant reduction in trypsin residual activity to approximately 21% (Figure_10a).

### Thermostability of rCcTI-epi-1 protein

The effect of different temperatures on residual inhibitory activity of rCcTI-epi-1 protein predicted moderate stability of inhibitor at higher temperatures. Up until 60°C of rCcTI-epi-1 treatment, the protein retained 75% of inhibitory activity against porcine trypsin which after 80°C showed a marked decline in the enzymatic activity to 34.56%. The inhibitory potential of rCcTI-epi-1 protein decreased stepwise in a linear manner with

Xi'an Shiyou Daxue Xuebao (Ziran Kexue Ban)/
Journal of Xi'an Shiyou University, Natural Sciences Edition
ISSN: 1673-064X
E-Publication: Online Open Access
Vol: 68 Issue 02 | 2025
DOI: 10.5281/zenodo.14842598

every increase in 10°C gradient in temperature. At 90°C, the thermal stability of rCcTI-epi-1 protein was 22.1% (Figure 10_b).

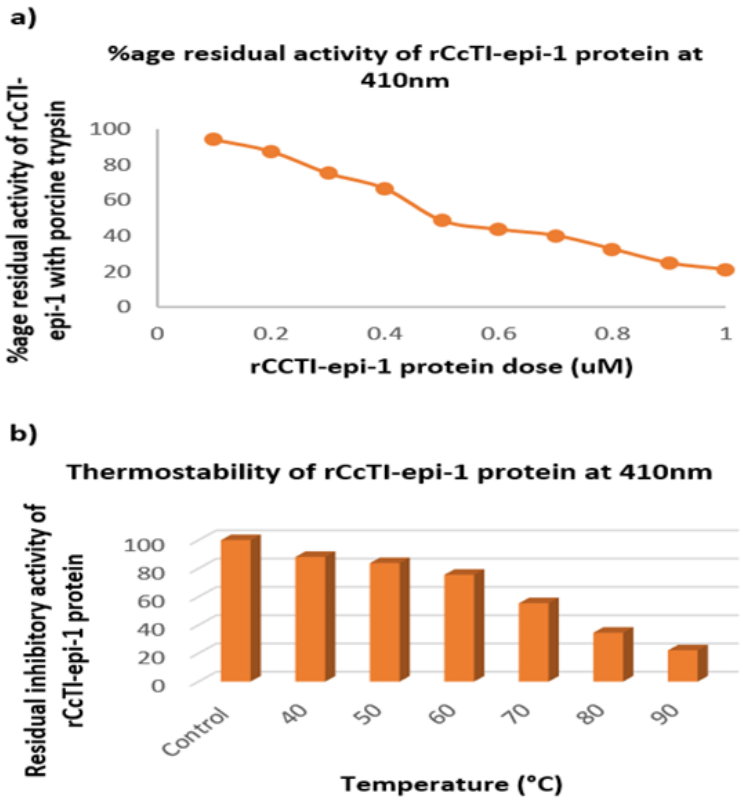



**Figure 9: Enzyme kinetics of rCcTI-epi-1 protein. a) Residual trypsin activity of rCcTI-epi-1 protein against porcine trypsin, b) Thermostability of rCcTI-epi-1 protein at different temperature (°C) conditions**

## Antimicrobial assay by agar diffusion method

CcTI protein as well as epinecidin-1 peptide have antibacterial activities against both gram-positive and gram-negative bacteria. Epinecidin-1 is referred to as AMP, an antimicrobial peptide found to exhibit bactericidal effect at a minimum dosage of 50µg/ml (Chee et al., 2019). This bacterial in vitro susceptibility potential of rCcTI-epi-1 protein caused the highest bacterial inhibition at a dose of 18µg/ml giving a 24mm zone of inhibition against *E. coli* (Figure_11a) while the maximum zone of inhibition (i.e. 18mm) against *S. aureus* was found with an intermediate dosage level of 36µg/ml (Figure_11b). Thus, fused trypsin inhibitor protein (rCcTI-epi-1) had a greater antimicrobial potential, owing to the excellent antimicrobial efficiency of the epinecidin-1 peptide partner. Trypsin inhibitor strongly inhibited the growth of *E. coli* while the bactericidal effect against *S. aureus* was mainly because of inhibitory action exerted by epinecidin-1 peptide, as the trypsin inhibitor has a little to no effect against it (Lin, Hui, Chen, & Wu, 2013).

**Xi'an Shiyou Daxue Xuebao (Ziran Kexue Ban)/**
**Journal of Xi'an Shiyou University, Natural Sciences Edition**
ISSN: 1673-064X
E-Publication: Online Open Access
Vol: 68 Issue 02 | 2025
DOI: 10.5281/zenodo.14842598

**Table 2: Antimicrobial activity of rCcTI-epi-1 protein against pathogenic bacterial strains.**

| | **Concentration** (µg/ml) | ***Escherichia coli*** | ***Staphylococcus aureus*** |
|---|---|---|---|
| **Antimicrobial potential of rCcTI-epi-1 protein** | 18µg/ml | 24mm | 12mm |
| | 36µg/ml | 16mm | 18mm |
| | 72µg/ml | 13mm | 12mm |

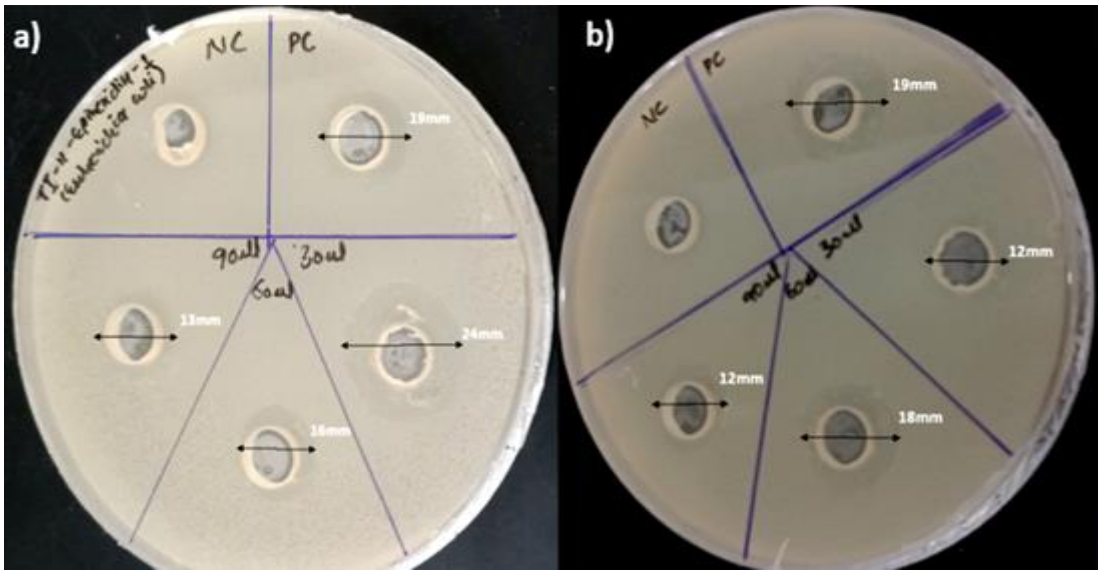



**Figure 10: Antimicrobial assay by disc diffusion method. The assay was carried in triplicates concentrations of rCCTI-epinecidin 1 protein treatment alongside with positive (amoxicillin) and negative control (Tris buffer pH 7.5) against two pathogenic bacterial strains. (a) rCCTI-epinecidin 1 protein against Escherichia coli (b) rCCTI-epinecidin 1 protein treatment against Staphylococcus aureus**

## Anti-cancer assay

Anti-angiogenic targeting of integrin $\alpha$VβIII, a precancerous receptor on cancer cells by anti-proliferative agents is a strategy to identify new onco-therapeutic compounds. In 2017, western blotting measured the expression levels of integrin $\alpha$VβIII on different cancer cells leading to the ascending order as MDA-MB-231>A549>HepG2 cells. The pattern of integrin expression observed in MCF-7 was the same as in MDA-MB-231 cancer cell lines (Ma et al., 2017). The anti-proliferative activity of rCcTI-epi-1 was assessed against 4 different human cancer cell lines i.e. MDA-MB-231, MCF-7, A549, and HepG2 cell lines using MTT assay. The dosage-dependent protocol for rCcTI-epi-1 protein was set in triplicates in 2 two-fold serial dilutions ranging from 2.25µg/ml - 300µg/ml.

**Xi'an Shiyou Daxue Xuebao (Ziran Kexue Ban)/**
**Journal of Xi'an Shiyou University, Natural Sciences Edition**
ISSN: 1673-064X
E-Publication: Online Open Access
Vol: 68 Issue 02 | 2025
DOI: 10.5281/zenodo.14842598

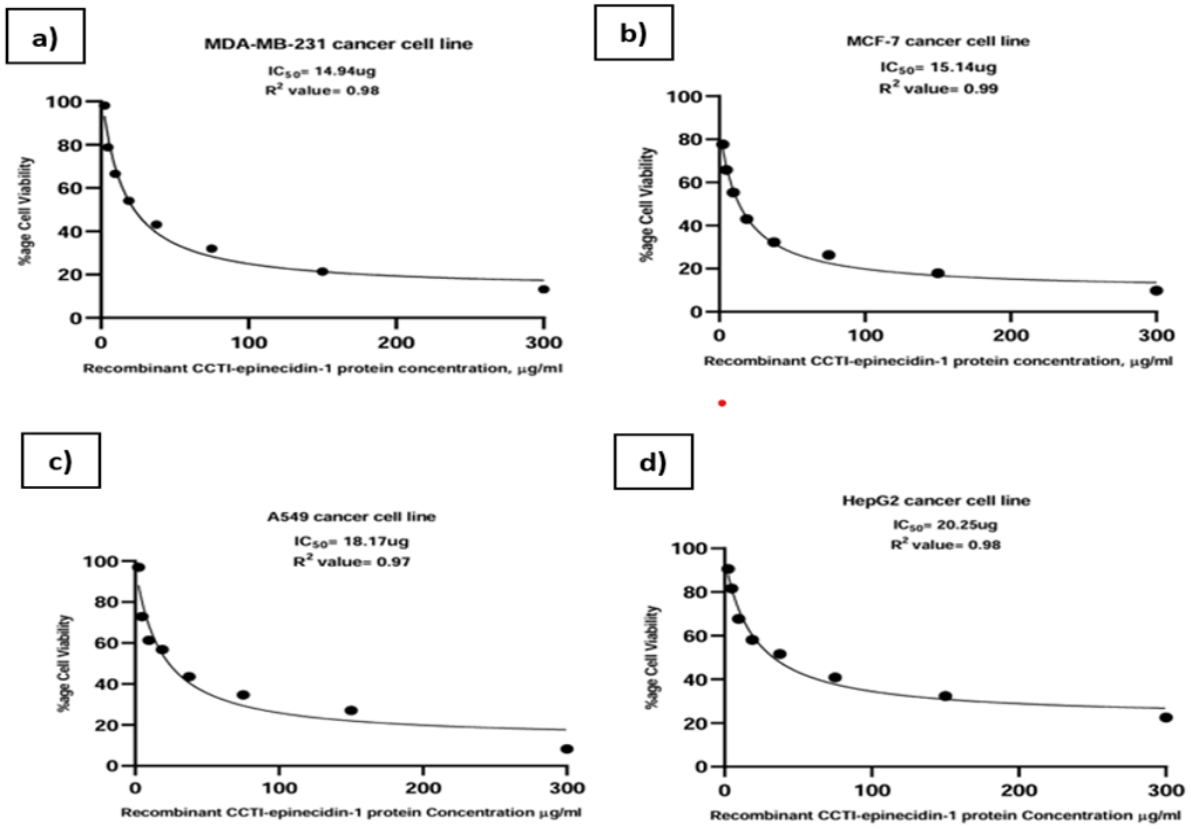



**Figure 11: Anticancer assessment of rCCTI-epinecidin-1 protein against the cell viability of a) MDA-MB-231, b) MCF-7, c) A549, and d) HepG2 cell lines**

With rCcTI-epi-1 protein, the IC50 values against MDA-MB-231, MCF-7, A549, and HepG2 cell lines were 15.86µg/ml, 17.33µg/ml, 18.17µg/ml, and 20.25µg/ml (shown in Figure_12). Our findings came in sync with previous facts suggesting that rCcTI-epi-1 protein strongly targeted integrin $\alpha$VβIII molecules on breast carcinoma and lung carcinoma showing a down-regulatory prognosis, while, a comparatively lower inhibitory effect was observed with hepatocellular cancer cells.

## CONCLUSION

Such an integrative work plan comprising of the insilico and invitro studies was carried out to investigate the biological potentials of purified recombinant *Cajanus cajan* with and without the inclusion of epinecidin-1 peptide. The rCcTI-epi-1 protein was purified from the *E. coli* expression system at a range of pH 7-8, while the inhibitory activity was retained at pH 4-10 ranging from both the acidic and alkaline nature of the protein. The cytotoxic action of rCcTI-epi-1 protein over integrin $\alpha$VβIII receptor was investigated by carrying out cytotoxicity assessment against human carcinoma cell lines bearing integrin receptors on their cells. Trypsin inhibitor is a cytotoxic protein with significant cytotoxic

ISSN: 1673-064X
E-Publication: Online Open Access
Vol: 68 Issue 02 | 2025
DOI: 10.5281/zenodo.14842598

effects against different human cancer cell lines. Additionally, inclusion of the fusion partner of epinecidin-1 with CcTI protein was effective enough to exert a stronger bactericidal and cytotoxic effects against pathogenic bacterial strains and human cancer cell lines. Thus, exploring the cost-effective synthesis and purification of *Cajanus cajan* trypsin inhibitor in fusion with epinecidin-1 emphasizes the biological potential of being developed as a potent commercial enterprise to produce rCcTI-epi-1 on large scale for future therapeutic applications for human complications.

**Funding:** This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

**Declaration of interest:** 'None'

**References**

1) Benchabane, M., Goulet, M.-C., Dallaire, C., Côté, P.-L., & Michaud, D, Hybrid protease inhibitors for pest and pathogen control–a functional cost for the fusion partners?, Plant Physiol Biochem. 46 (2008) 701-708.

2) Bhatt, H. B., & Singh, S. P.,. Cloning, expression, and structural elucidation of a biotechnologically potential alkaline serine protease from a newly isolated haloalkaliphilic Bacillus lehensis JO-26, Front. microbiol. 11 (2020) 941.

3) Bhattacharjee, N., Banerjee, S., & Dutta, S. K., Cloning, expression and mutational studies of a trypsin inhibitor that retains activity even after cyanogen bromide digestion, Protein Expr. Purif. 96 (2014) 26-31.

4) Chee, P. Y., Mang, M., Lau, E. S., Tan, L. T.-H., He, Y.-W., Lee, W.-L., . . . Goh, B.-H., Epinecidin-1, an antimicrobial peptide derived from grouper (Epinephelus coioides): Pharmacological activities and applications, Front. microbiol. 10 (2019) 2631.

5) Cisneros, J. S., Cotabarren, J., Parisi, M. G., Vasconcelos, M. W., & Obregón, W. D, Purification and characterization of a novel trypsin inhibitor from Solanum tuberosum subsp. andigenum var. overa: Study of the expression levels and preliminary evaluation of its antimicrobial activity, Int. J. Biol. Macromol. 158 (2020) 1279-1287.

6) Cotabarren, J., Lufrano, D., Parisi, M. G., & Obregón, W. D, Biotechnological, biomedical, and agronomical applications of plant protease inhibitors with high stability: A systematic review, Plant Sci, 292 (2020) 110398.

7) Davis, R. L., Harvey, M. C., Watson, N., Mehigh, R. J., Dapron, J. G., Chen, D. E., & Scott, G. B. One-Step Purification of Histidine-Tagged Fusion Proteins Under High Pressure and High Flow Conditions.

8) do Amaral, M., Freitas, A. C. O., Santos, A. S., Dos Santos, E. C., Ferreira, M. M., da Silva Gesteira, A., . . . Pirovani, C. P, TcTI, a Kunitz-type trypsin inhibitor from cocoa associated with defense against pathogens, Sci. Rep. 12 (2022) 698.

9) Habib, H., Zargar, M. A., & Fazili, K. M. Cloning and Expression of Trypsin Inhibitor Gene Ti from Pea (Pisum sativum L.) cv.'Arkel'in Escherichia coli DH5 Cells.

10) Hong, T. T., Dat, T. T. H., Hoa, N. P., Dung, T. T. K., Huyen, V. T. T., Cuc, N. T. K., & Cuong, P. V, Expression and characterization of a new serine protease inhibitory protein in Escherichia coli, Biomed Res Ther. 7 (2020) 3633-3644.

ISSN: 1673-064X
E-Publication: Online Open Access
Vol: 68 Issue 02 | 2025
DOI: 10.5281/zenodo.14842598

11) Jeyarajan, S., Peter, A. S., Sathyan, A., Ranjith, S., Kandasamy, I., Duraisamy, S., . . . Kumarasamy, A, Expression and purification of epinecidin-1 variant (Ac-Var-1) by acid cleavage, Appl. Microbiol. Biotechnol., 108 (2024) 1-15.

12) Khakha, S., Sharma, A., Kumari, P., Sahi, S., & Biswas, S, In-silico molecular characterization and mutational analysis of inter-alpha-trypsin inhibitor heavy chain 4 in rheumatoid arthritis, J. Pro. Proteo. 10 (2019) 313-323.

13) Kielkopf, C. L., Bauer, W., & Urbatsch, I. L, Purification of polyhistidine-tagged proteins by immobilized metal affinity chromatography, Cold Spring Harb. Protoc. (2020) pdb. prot102194.

14) Krishnan, B., Hedstrom, L., Hebert, D. N., Gierasch, L. M., & Gershenson, A, Expression and purification of active recombinant human alpha-1 antitrypsin (AAT) from Escherichia coli, Alpha-1 Antitrypsin Deficiency: Methods and Protocols, (2017) 195-209.

15) Kwon, C. W., Chung, B., Yoo, S.-H., & Chang, P.-S, Heterologous expression of a papain-like protease inhibitor (SnuCalCpI17) in the E. coli and its mode of inhibition, Appl. Microbiol. Biotechnol. 106 (2022) 4563-4574.

16) Laskowski, M., & Laskowski Jr, M, Naturally occurring trypsin inhibitors, Adv. Protein Chem. 9 (1954) 203-242.

17) Lin, M.-C., Hui, C.-F., Chen, J.-Y., & Wu, J.-L, Truncated antimicrobial peptides from marine organisms retain anticancer activity and antibacterial activity against multidrug-resistant Staphylococcus aureus, Peptides. 44 (2013) 139-148.

18) Ma, Y., Ai, G., Zhang, C., Zhao, M., Dong, X., Han, Z., . . . Gao, W, Novel linear peptides with high affinity to αvβ3 integrin for precise tumor identification, Theranostics. 7 (2017) 1511.

19) Malik, A, Protein fusion tags for efficient expression and purification of recombinant proteins in the periplasmic space of E. coli, *3 Biotech*. 6 (2016) 44.

20) Mehmood, S., Imran, M., Ali, A., Munawar, A., Khaliq, B., Anwar, F., . . . Saeed, A, Model prediction of a Kunitz-type trypsin inhibitor protein from seeds of Acacia nilotica L. with strong antimicrobial and insecticidal activity, Turk J Biol. 44 (2020) 188-200.

21) *Microsoft Word - pET Manual 8th 0199pc.doc*. (7/22/14). Acrobat Distiller 3.01 for Power Macintosh.

22) Neshani, A., Zare, H., Akbari Eidgahi, M. R., Khaledi, A., & Ghazvini, K, Epinecidin-1, a highly potent marine antimicrobial peptide with anticancer and immunomodulatory activities, BMC Pharmacol Toxicol. 20 (2019) 1-11.

23) Pirovani, C. P., da Silva Santiago, A., Dos Santos, L. S., Micheli, F., Margis, R., da Silva Gesteira, A., . . . de Mattos Cascardo, J. C, Theobroma cacao cystatins impair Moniliophthora perniciosa mycelial growth and are involved in postponing cell death symptoms, Planta. 232 (2010) 1485-1497.

24) Samavarchi Tehrani, S., Gharibi, S., Movahedpour, A., Goodarzi, G., Jamali, Z., Khatami, S. H., ... & Taheri-Anganeh, M., Design and evaluation of scFv-RTX-A as a novel immunotoxin for breast cancer treatment: an in silico approach, J Immunoassay Immunochem. 42 (2021) 19-33.

25) Vandierendonck, J., Girardin, Y., De Bruyn, P., De Greve, H., & Loris, R, A multi-layer-controlled strategy for cloning and expression of toxin genes in Escherichia coli, Toxins. 15 (2023) 508.

26) Völzke, J. L., Smatty, S., Döring, S., Ewald, S., Oelze, M., Fratzke, F., . . . Weller, M. G, Efficient Purification of Polyhistidine-Tagged Recombinant Proteins Using Functionalized Corundum Particles, BioTec. 12 (2023) 31.