# Structural and Biophysical Characterization of *Cajanus cajan* Protease Inhibitor

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### Abstract

**Context:** A large number of studies have proven that Protease inhibitors (PIs), specifically serine protease inhibitors, show immense divergence in regulation of proteolysis by targeting their specific proteases and hence, they play a key role in healthcare. **Objective:** We aimed to access *in-vitro* anticancer potential of PI from *Cajanus cajan* (CCPI). Also, crystallization of CCPI was targetted alongwith structure determination and its structure-function relationship. **Materials and Methods:** CCPI was purified from *Cajanus cajan* seeds by chromatographic techniques. The purity and molecular mass was determined by SDS-PAGE. Anticancer potential of CCPI was determined by MTT assay in normal HEK and cancerous A549 cells. The crystallization screening of CCPI was performed by commercially available screens. CCPI sequence was subject to BLASTp with homologous PIs. Progressive multiple alignment was performed using clustalw2 and was modelled using *ab initio* protocol of I-TASSER. **Results:** The results showed ~14kDa CCPI was purified in homogeneity. Also, CCPI showed low cytotoxic effects of in HEK i.e., 27% as compared with 51% cytotoxicity in A549 cells. CCPI crystallized at 16°C using 15% PEG 6000 in 0.1M potassium phosphate buffer (pH 6.0) in 2-3weeks as rod or needles visualized as clusters under the microscope. The molecular modelling revealed that it contains 3 beta sheets, 3 beta hairpins, 2 β-bulges, 6 strands, 3 helices, 1helix-helix interaction, 41 β-turns and 27 γ-turns. **Discussion and Conclusion:** The results indicate that CCPI may help to treat cancer *in vivo* aswell. Also, this is the first report on preliminary crystallization and structural studies of CCPI.

Keywords: Anticancer, Cajanus cajan, crystallization, homology modeling, protease inhibitor, sequence analysis

## INTRODUCTION

Protein protease inhibitors (PPIs) are proteins that diminish the proteolytic activity of proteases. They form a stable complex with target proteinase by either altering, blocking, or preventing access to the active site of enzyme and hence play important role in regulation of proteolysis.<sup>[1]</sup> PPIs are omnipresent and versatile and hence are used in a wide range of field including their role as therapeutic agents in diseases, specifically cellular transformation, osteoporosis, blood clotting disorders, retroviral disease, cancer, etc., Currently, PPIs are keenly investigated for their role as anticancer agents, i.e., inhibition of transformed cell growth.<sup>[2-5]</sup> Metastasis of cancer cells requires action of the matrix metalloproteinases and serine proteases that constitute a complex interacting protease cascade system. Hence, inhibition of such processes and proteins is most likely to be the molecular targets for cancer prevention.<sup>[6]</sup> Furthermore, studies are being performed

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to investigate protease inhibitors (PIs) as novel drugs in highly active antiretroviral combination therapy, which aim to increase life expectancy of an HIV-positive patient.<sup>[7,8]</sup>

In field of agriculture, to counterbalance the loss caused by chemical pesticides, plant PPIs have gained remarkable attention as natural defense agents in plants.<sup>[9]</sup> Along with the role of growth inhibition of insects and pests, PPIs also show inhibitory activity of pathogenic nematodes such as *Globodera tabaccum*<sup>[10]</sup> and pathogenic fungi such as *Trichoderma reesei*<sup>[11]</sup> and *Alternaria alternata*.<sup>[12]</sup> With the wide range of applications in the field of medicine and agriculture, researchers have gained a keen interest in searching novel PPIs and their therapeutic

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importance.<sup>[13]</sup> Therefore, a number of PPIs have been isolated and characterized from various plant sources.<sup>[14-20]</sup>

Plants have ability to produce certain biologically active compounds that are believed to be involved in the defense mechanism against pests, insects, and microbial attacks. This system includes use of defense proteins such as PIs, lectins, amylase inhibitors, and few pathogenesis-related proteins.[21-24] To be specific, pigeon pea (Cajanus cajan L.) seeds contain PIs of trypsin, chymotrypsin, and amylases, [25,26] as well as secondary metabolites and phytolectins,<sup>[27,28]</sup> as the defense machinery against pest and microbial infection. Pichare and Kachole have reported seven isoforms of trypsin-chymotrypsin inhibitors and two isoforms of trypsin inhibitors (TIs) from C. cajan seeds.<sup>[29]</sup> In addition, Godbole et al. and Haq and Khan depicted C. cajan PI (CCPI) as Kunitz-type PI having inhibitory activity against trypsin and chymotrypsin.<sup>[17,30]</sup> Our study aimed to purify CCPI from the seeds of C. cajan and its role in cellular cytotoxicity in normal and cancer cell line was assessed. For the first time, crystallization study of CCPI was performed and its in-silico analysis was done to support the structure-function relationship.

## **MATERIALS AND METHODS**

#### Chemicals, reagents, and materials

*C. cajan* (PUSA-992 variety), commonly known as "*arhar*," was received from IARI, New Delhi. Chemicals: trypsin (bovine pancreatic trypsin), N $\alpha$ -benzoyl-DL-argi nine-4-nitroanilide hydrochloride (BAPNA), acrylamide, tetramethylethylenediamine, bis-acrylamide, and ammonium persulfate were obtained from Sigma-Aldrich. For an initial screen, commercially available crystallization screens were purchased from Molecular Dimensions. All other reagents and chemicals used were of analytical grade.

#### **Cell lines and cell culture**

Human embryonic kidney (HEK) and adenocarcinomic human alveolar basal epithelial cells (A549) were maintained in RPMI-1640 grown in 10% fetal bovine serum (FBS) and antibiotics (100 U/ml penicillin and 100  $\mu$ g/ml streptomycin). The cells were cultured at 37°C, 5% CO<sub>2</sub> humid condition in CO<sub>2</sub> incubator (Thermo). All the other chemicals were procured from Sigma-Aldrich and Merck.

#### Purification of Cajanus cajan protease inhibitor

CCPI was purified as per the protocol mentioned by Haq and Khan<sup>[17]</sup> Fractions showing inhibitory activity against trypsin were pooled together, dialyzed against Tris buffer (pH 8.2) for desalting, and loaded onto fast protein liquid chromatography (FPLC) gel filtration Superdex-75 column preequilibrated with Tris buffer (pH 8.2). CCPI was eluted at the rate of 0.5 ml/min. The purified and active fractions of CCPI were taken as sample for further research.

# Determination of purity and molecular weight by sodium dodecyl sulfate-polyacrylamide gel electrophoresis

The purity and molecular mass of CCPI were determined by sodium dodecyl sulfate-polyacrylamide

gel electrophoresis (SDS-PAGE) performed on 12% polyacrylamide slab gel under reducing conditions using method of Laemmli.<sup>[31]</sup> CCPI sample was mixed with a sample buffer (0.125-M Tris-HCl pH 6.8, 20% glycerol, 4% SDS, and 10%  $\beta$ -mercaptoethanol) in equal ratio. The mixture was then brought to boiling for 5 min. Fifteen microliters of protein sample was loaded onto gel composed of 5% stacking gel and 12% resolving gel. The electrophoresis was carried out at constant 100V current using Mini-Protean apparatus (Bio-Rad). After electrophoresis, gel was stained with 0.1% Coomassie Brilliant blue R-250 of water:methanol:acetic acid in 50:40:10 for 1 h and destained with solution of water: methanol:acetic acid in 50:40:10 overnight. The molecular weight of CCPI was estimated by comparing with Puregene prestained Protein Ladder, Broad Range (10–250 kDa).

#### Evaluation of trypsin-inhibitory activity

The enzymatic activity of CCPI against trypsin was checked as per the protocol of Erlanger *et al.* with minor modifications.<sup>[32]</sup> The residual enzymatic activity was checked using BAPNA-HCl as substrate. Twenty microliters of trypsin (1 mg/m1) was incubated with 100  $\mu$ l of CCPI sample and 80  $\mu$ l of Tris buffer (pH 8.2) for 10 min at room temperature. The reaction was initiated by addition 500  $\mu$ l BAPNA solution (1.5 mM) and was incubated at room temperature. Three hundred microliters of 30% acetic acid solution was added after 10 min to terminate the reaction. The total reaction mixture volume was 1 ml. The decrease in intensity of yellow color due to enzymatic hydrolysis of the BAPNA was visualized at 410 nm, which corresponded to release of p-nitroaniline and hence the TI activity.

#### Assessment of cytotoxic activity against cancer cell line

Anti-proliferative activity of CCPI was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay using the protocol of Verma et al.[33] Anti-proliferative studies of CCPI were performed using MTT assay on adenocarcinomic human alveolar basal epithelial cells (A549) and nontumor HEK cell lines, respectively. Cells were seeded at the density of  $5 \times 10^3$  cells/well in a 96-well plate supplemented with 2.5% FBS. After treatment with the PPIs, cells were incubated at 37°C with two different concentrations, i.e., 10 µg/ml and 5 µg/ml for 24 and 48 h. After the necessary time period, 20 µl of MTT solution (5 mg/ml in PBS buffer, pH 7.4) was added to wells and incubated for 4 h. After adding 150 µl of dimethyl sulfoxide that dissolves formazan crystals formed from cellular reduction of MTT in well, the plate was read at the optical density of 540 nm wavelengths on the ELISA-reader (Synergy HT, Biotek, USA).

All measurements were done in triplicates. The percent cytotoxicity values were determined by:

% cytotoxicity =  $([A]_{control} - [A]_{test})/(A)_{control} \times 100$ 

Where  $(A)_{control}$  is the absorbance of control sample and  $(A)_{test}$  is absorbance of test sample.

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# Screening and developing *Cajanus cajan* protease inhibitor crystals

For an initial screen, commercially available screens were used that exploit the trial conditions. These screens included (1) PACT premier; (2) JCSG plus; (3) Morpheus; (4) Proplex; (5) Three-dimensional (3D) structure; (6) MacroSol; (7) PGA screen; (8) Structural screen. CCPI sample was spun at 15 min/18,000  $\times$ g/4°C to settle down dust and aggregated proteins (if any). The sample was concentrated to the final concentration 5 mg/ml using an Amicon filter. The 96-well hanging drop tray was filled with 100 µl of reservoir buffers according to the tray setup scheme as per screen kits. Ten microliters of CCPI was loaded for each row of well on the cover slide which was then distributed in all wells using mosquito pipetting robot (TTP Labtech). The cover slide was flipped gently and laid down on the grease ring on top of the well. The slide was pressed gently to allow the air entrapped to escape and keep the well sealed. The trays were incubated at 16°C undisturbed and were observed regularly under microscope to visualize the crystal formation.

#### **Primary X-ray diffraction analysis**

For initial characterization, CCPI crystals were observed under an R-AXIS IV++ image-plate detector and Rigaku rotating-anode X-ray generator at room temperature using Cu K-radiation.

#### Sequence analysis and annotations

CCPI sequence retrieved from the UniProt database (Uniprot: Q5U9N0) (http://www.uniprot.org/). This sequence information was analyzed to determine regulatory sequences, structural motifs, and repetitive sequences. A comparison of genes within a species or between different species can show either similarities between protein functions or relations between species. The CCPI sequence was subject to BLASTp with homologous PIs from different species. Progressive multiple alignment was performed using clustalw2.

#### Homology modeling

CCPI (Uniprot: Q5U9N0) was modeled using *ab initio* protocol of the I-TASSER.<sup>[34]</sup> Subsequently, five models were generated and assessed on the basis of RMSD and TM-score. This online server theoretically measures various physicochemical parameters such as molecular mass. The overall quality factor score of CCPI was predicted by ERRAT (http://nihserver. mbi.ucla.edu/ERRAT/). The refined structure was validated using SAVES (http://services.mbi.ucla.edu/SAVES/). The topological analysis of the given CCPI structure was done using PDBsum, for understanding the structural features of CCPI structure in detail.

## **Results and Discussion**

#### Importance of Cajanus cajan protease inhibitor

CCPI has been found to be of importance in plant defense. Various studies performed *in-vitro* and *in-vivo* have suggested that the CCPI are potentially active against proteases of larval guts, which lead to impaired digestion and amino acid absorption, cause retarded growth and development of larvae, and lead to loss of fertility and productivity of adult moths. For instance, CCPI shows moderate inhibition potential toward insect *Helicoverpa armigera* gut proteinases.<sup>[35]</sup> It also diminished the activity of proteinases of larval midgut showing trypsin-like nature in *Manduca sexta*.<sup>[36]</sup> CCPI being smaller in size can be expressed in castor plants to protect them against their lethal pest *Achaea janata* by inhibiting its midgut trypsin-like proteases.<sup>[37]</sup>

# Purification and characterization for activity, molecular weight, and purity

CCPI was isolated and purified in homogeneity from C. cajan seeds. It involved three-step chromatography method which included double ion-exchange (from previous study) and FPLC on Superdex 75 column where an elution profile was obtained [Figure 1a]. The CCPI obtained was subjected to protein concentration determination and TI activity assay at each step [Table 1]. Although PPIs from C. cajan have earlier been purified, the family or type of inhibitor to which they belong does not depict a clear picture. Godbole et al. purified two PIs kDa from C. cajan cv. TAT-10 showing molecular weight of ~15 and ~10.5 and proposed that the PPI belonged to Bowman-Birk inhibitor (BBI) family.<sup>[30]</sup> However, as we know, PIs belonging to BBI family have lower molecular weight of around 6-9 kDa.<sup>[38]</sup> Furthermore, Haq and Khan purified CCPI of molecular weight ~14 kDa and concluded on basis of its N-terminal sequence that CCPI belonged to Kunitz family.<sup>[17]</sup> Further, Osowole et al. isolated PPI from C. cajan weighing ~18.2 kDa.<sup>[39]</sup> Norioka et al. showed that CCPIs are only BBI on the basis of gel filtration peaks.<sup>[40]</sup> Further, Prasad et al. purified BBI-type PI from C. cajan naming it Red Gram PI.<sup>[36]</sup> Our protocol resulted in purification of CCPI



**Figure 1:** (a) Fast protein liquid chromatography chromatogram of *Cajanus cajan* protease inhibitor eluted on Superdex 75 column. The graph represents concentration of *Cajanus cajan* protease inhibitor eluted on Y axis with eluted fraction on Y axis. (b) Electrophoretogram of purified *Cajanus cajan* protease inhibitor on sodium dodecyl sulfate polyacrylamide gel electrophoresis (12%) shows molecular-weight markers (Lane 1) and a single band of molecular mass 14 kDa eluted by fast protein liquid chromatography on Superdex 75 column (Lane 2)

with homogeneity which was depicted as a thick single band at 14 kDa investigated on 12% SDS-PAGE [Figure 1b].

#### Assessment of cytotoxic activity against cancer cell line

Our results clearly proved that the CCPI was found active against tumor cells when compared to nontumor cells in time- and concentration-dependent manner. CCPI was added in two different concentrations and the cells were incubated for two different time intervals and cytotoxic effect of CCPI on cells was accessed. MTT end-points suggested that the IC<sub>50</sub> value of CCPI for A549 cells was ~9.84  $\mu$ g/ml which was lower as compared to IC<sub>50</sub> value for HEK cells, i.e., ~18.18 µg/ml. CCPI showed low cytotoxic effects in HEK (27%) than A549 cells (51%) at 48 h, which were higher to the values obtained at 24 h interval. Hence, we can conclude that CCPI shows higher cytotoxicity against A549 cells as compared to HEK cells in time- and concentration-dependent manner [Figure 2]. Rakashanda et al. reported the IC<sub>50</sub> values of Lavatera cashmeriana PIs to be  $36 \pm 2 \mu g/ml$  in human lung cancer cell line (NCIH322), which was quite higher than the results obtained in our studies.<sup>[6]</sup> Hence, CCPI demonstrates more inhibitory effect on cancer cell lines and therefore can be depicted as an antitumor drug in near future.

#### Screening and crystallization

CCPI was screened preliminarily with seven different screens. Morpheus and 3D structural screen were repeated.



**Figure 2:** Cytotoxic effect of *Cajanus cajan* protease inhibitor on A549 and human embryonic kidney cell lines. Results are depicted as bar diagrams. The cells were incubated with *Cajanus cajan* protease inhibitor at 5  $\mu$ g/ml and 10  $\mu$ g/ml in a 96-well plate. Optical density at 540 nm was measured after 24 and 48 h and percentage cytotoxicity was determined

The conditions showing sign of crystal growth were repeated manually on a 24-well plate. Crystals of the CCPI were obtained in 2–3 weeks using 15% polyethylene glycol (PEG) 6000 in 0.1M potassium phosphate buffer, pH 6.0 [Figure 3]. The crystals obtained were either rectangular- or rod-like structure grouped in the form of clusters which was quite similar to the orthorhombic crystals of PI from *Tamarindus indica*.<sup>[20]</sup> Due to lack of symmetry and homogeneity, the crystals of CCPI could not diffracted by X-ray. Therefore, the size of crystals could not be determined, but this was the first study in the context of crystallization of CCPI till date.

#### Sequence and structure determination

The CCPI sequence obtained from http://www.uniprot.org/ (Uniprot: Q5U9N0) has 176 amino acid sequences; first 1–19 are the signal peptide shown in red and 20–176 are chain [Figure 4a]. The molecular mass of *C. cajan* was 19.97 kDa with isoelectric point 9.54.<sup>[41]</sup> Procheck showed that 77.6% of the residues were in the allowed region of Ramachandran plot. The overall quality factor score predicted by ERRAT was 69.04 for PI. PDBsum showed that initial CCPI contained strands 7.4% (13 aa), alpha helix 11.4% (20 aa), and other 81.2% (143 aa). The structure showed the presence of 3 beta-sheets, 3 beta-hairpins, 2  $\beta$ -bulges, 6 strands, 3 helices, 1 helix–helix interaction, 41  $\beta$ -turns, and 27  $\gamma$ -turns. Moreover, there was no disulfide bonds were found in the structure of *C. cajan* [Figure 4b].<sup>[42]</sup>



**Figure 3:** The purified *Cajanus cajan* protease inhibitor was crystallized by hanging drop technique using 0.1M potassium phosphate buffer pH 6.0 and 15% (w/v) polyethylene glycol 6000 within 2–3 weeks. The crystals were visualized under high resolution microscope

Table 1: Purification profile of Cajanus cajan protease inhibitors and its activity at each step											
Step	Protein concentration (mg/ml)	Amount (ml)	Total protein (mg)	Yield (%)	Activity (U)	Specific activity (U/mg)	Purification (fold)				
Homogenate	3.2	120	384	100	2,539,298.66	6612.756927	1				
$30\%-50\% (NH_4)_2 SO_4$ precipitate	3.027	60	181.62	37.69	1,176,541.72	6478.04	1.2				
First eluent	1.8	40	72	14.94	530,108.83	7362.62	1.4				
Second eluent	1.2	30	36	7.47	313,108.83	8697.46	1.66				
FPLC eluent	0.8	30	24	6.25	350,120.91	14,588.37	2.21				

FPLC: Fast protein liquid chromatography

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		de 19 176	β <u>MMV</u> 1 A - CRI 61 γ	ββ β <u>LKVC</u> 5 <u>γ</u> <u>γ</u> NHIG	ββ ββ <u>VIVLF</u> 10 β β SH3KG 70 ββ	β γ γ <b>INGVI</b> 15 <b>ΝΤΓΚΥ</b> 75 ββββ	β ββ <u>TANMR</u> 20 γ LAIIT 80 H3	γβ <u>MPEIR</u> 25 β <u>PIGNE</u> 85	γγγ <b>GVWKV</b> 30 βββββ SV PHEM 90 γ	H1 EYLE 35 βββ MFRT 95 B	βγ         β           40           βββ         γ           βββ         γ           100         C           γ         γ	<ul> <li>β β β γ</li> <li>8</li> <li>9</li> <li>9</li></ul>	xAVPT 50 β TIGILI 110 C	γ γ β <u>RKPVN</u> 55 H2 <u>H2</u> <u>H3</u> <u>H3</u> <u>H15</u>	β <u>₩QPI</u> 60 <u>₩QPI</u> 120
2			NSI	SFKI	ICLSS	IEHFI	ISHLK	NVYM(	VGMRV			PHIS	STYL	LEIKI	
Sequence	Position	Description	121	123	C	ell	140	140	150	Pa	ittern	105	1/0	1/5	
					С	ompa	irtme	nt							
TYVDA	99-103[A]	Caspase3 And caspase7			C	ytosol	, nucle	eus		[D	STE][/ DFWH	·P] FYC1D	GSA	1	
TRK	51-53[A]	NArg dibasic		E) aj	Extracellular,golgi apparatus, cell surface				(.R	(.RK)(RR[^KR])					
TRK	74-76[A]	Convertase (NRD/ Nardilysin) cleavage s RIRX).	Convertase (NRD/ Nardilysin) cleavage site (X RKor				Golgi apparatus, cell surface			(.R	(.RK) <b> </b> (RR[^KR])				
TRKYLAIIT	74-82[A]	An RxxL based motif that binds to the Cdh1 and Cdc20 components of APC/C thereby targeting the protein for destruction in a cell cycle dependent				Nucleus, cytosol			.RL[LIVM].						
TRKPVNWQ	51-58[A]	manner Protein phosphatasel catalytic subunit (PP1c) interacting motif binds targeting proteins that dock to the substrate for dephosphorylationThe motif defined is [RK]0,1][V1][^P] [FWI				Nucleus, protein phosphatas <del>a</del> ype 1 complex, cytosol			[RK].{0,1}[VIL][^P][FW].						
NSISF	121-125[A]	Phosphopeptide motif which directly interacts with the BRCT (carboxyterminal) domain of the Breast Cancer Gene BRCA1 with low affinity				Nucleus, BRCA1BARD1 complex			.(S	.(S)F					
NITGILH	106-112[A]	Phosphothreonine			N	ucleu	5			(1	r)[ILV	].			
PCTYVDA	97-103[A]	Phosphothreonine motif bin of FHA domains that have a for an acidic amino acid at th position.	Phosphothreonine motif binding a subse of FHA domains that have a preference for an acidic amino acid at the pT+3 position.				Nucleus, Replication fork			(1	(T)[DE].				
SFKII	124128[A]	Canonical LIR motif that bind protein family members to n processes involved inautoph	Canonical LIR motif that binds to Atg8 protein family members to mediate processes involved inautophagy				Cytosol, cytoplasmic side of late endosome membrane			[EDST].{0,2}[WFY][ILV]					
LLEIKI	171176[A]	The Cterminal class 2 PDZbin motif is classically represent pattern such as (VYF)X(VIL)*	nding ed by	y va	C p	ytosol lasma	, interi memt	nal sio prane	le of	[	VLIFY]	.[ACVI	LF]\$		

**Figure 4:** (a) Three-dimensional structure of *Cajanus cajan* protease inhibitor with 3  $\alpha$ -helices, and 3  $\beta$ -sheets. (b) The topology map of *Cajanus cajan* protease inhibitor generated by PDBsum showing secondary structure elements in the framework. (c) Representation of functional motifs present in *Cajanus cajan* protease inhibitor with patterns and position found in the query sequence

## **Homology modeling**

Multiple Sequence Alignment of CCPI is shown in Figure 5 where less conserved and highly conserved residues are highlighted in light and dark gray, respectively, while the conserved cysteine residue as highlighted in yellow.<sup>[43,44]</sup> The motif presents in CCPI by Eukaryotic Linear Motifs resource for the functional sites in proteins. In a protein, the motifs are key signatures of protein families and can be preferably used to define the protein function [Figure 4c].<sup>[45]</sup>

## CONCLUSIONS

We have purified ~10 mg CCPI from 100 g seeds of *C. cajan*. The results suggested that CCPI showed low cytotoxic effects of in HEK, i.e., 27% as compared in adenocarcinomic human alveolar basal epithelial A549 cells with 51% cytotoxicity. The CCPI protein was crystallized in 0.1M potassium phosphate buffer, pH 6.0, and 15% (w/v) PEG 6000 conditions in the interval of 2–3 weeks. The crystals developed were rod-shaped but could not be diffracted due to some reasons. The CCPI

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trio1WAK31	MMUT	KVCVLVVFLV	GVTAB-GMDL	NHLRSIH	HH-HDSSDEP	SESSEPCCDS	CRCTKSI	PROCECT
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tr[Q1WA44]	MMVI	KVCVLVVFLV	GVTTB-GMDL	NQLRSS-	HH-HDSSDEP	SESSERCCDS	CRCTKSI	PEQCECJ
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trIC6SX261	MGLKNNMVVL	KVCFLVLFLV	GVINA-RMEL	NLEKS-	DH-SSSDD	-ESSKPCCDL	CMRTASM	FROCHCI
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UT AVAUBLE	POOT LOUIS A A T	trade nonema	OV INS- HALLE	NDE	DH-333DD	-LOSNECCDL	CACIASM	20000000
tr   Q93WK3	MVVE	KVCFMLLFLL	GISTA-SLRM	SELGLRFKS-	GH-HOSTDEP	SESSKECCDH	CACTKSI	PROCECT
sp[P01060]	MMVL	RVGLLLVFLA	GVTTA-RMDL	NHLIGS-	NH-HDSSDEP	SESSERCCDI	CVCTASI	FFICOC1
++1177C6C31	TTRACT	SPERAT T. T. STORE 3.	TTTT - DMDT	MRT TCS-	MH-HDSSDED	SPECTOCOT	CUCTO ST	TRT COM
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tr A4Q9H5	MMVI	KVGLELVFEV	OVTIN-RMDL	NHLIRS-	NH-HDSSDEP	SESSERCCDL	CMCTDSI	PRICECI
tr1A7D2061	MMVL	KVGLLLVFLV	GVTTA-RMDL	NHLIRS-	NH-HDSSDEP	SESSERCCDH	CMCTDSI	PPICOCI
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tr[A40975]	MMVE	KAGTUPALEA	GVTTR-RMDL	NHLIR	NH-HDSSDEP	SESSERCCDL	CMCTHSI	PRICECI
tr   A8KRM8	MMVL	KVGLLLVFLV	GVTTR-RMDL	NHLIRG-	NH-HDSSDEP	SESSEBCCDL	CVCTDSI	PPICOCI
TYLASKEN41	MURIT	KUCT LLUFTU	GUTTE-PMDT	NHT TRG-	NH-HDSSDED	SESSEPCODT.	CVCTDST	DET COM
CT PROPERTY	1.11	CALO DUDALE NY	VIII PALO	1111	MA-NDOODLE	SESSECCEL	01010-01	
tr  B1GYE5	WMVD	RVGLLLIFEV	GVTTR-RMDL	NHLIRS-	NH-HDSSDEP	SESSERCCDL	CMCADSI	PRICECI
tr G912Q3	MMVI	KVCVLVVFLL	GVTAR-GMDL	NHLRSI-	HHNHDSSDEP	SESSEBCCDS	CRCTKSI	FROCECJ
++12020F3T		STATUTATURET.	OUT2 - CMDT	NHT DST-	HHNHDSSDED	SPREEDCODS	CPCTRST	DECCHO?
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tr   D6MZ10	MMVE	KAGATAATTAA	GVTTR-GMDL	NQLRSS-	HH-HDSSDEP	SESSERCCDS	CRCTKSI	EBOCHC1
tr1093YY21	MMVL	KVCVLVLFLV	GVTTA-AMDL	NHLGSN-	HH-DDSSDEP	SESSECCDS	CICTKSI	PBOCEC1
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**101WA441				RLNS	CHSACKS	CMCTREMPGK	CRCLDIAD-F CRCLDTDD-F	CYRPCE:
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tr   H6WNA3				RLNS RLNS	CHSACKS CHSACKS CHSACKS	CMCTRSMPGK CMCTRSMPGK CMCTRSMPGK CVCTFSIPAO	CRCLDIAD-F CRCLDIDD-F CRCLDIDD-F CVCVDMKD-F	CYKPCE: CYKPCE: CYKPCE:
tr  H6WNA3				RLNS RLNS RLNS	CHSACKS CHSACKS CHSACKS CHSACSS	CMCTREMPGK CMCTREMPGK CVCTFSIPAQ	CRCLDIAD-F CRCLDTDD-F CRCLDTDD-F CVCVDMKD-F	CYKPCE: CYKPCE: CYEPCK-
tr   H6WNA3   tr   Q941H6				RLNS RLNS RLNS RLNS	CHSACKS CHSACKS CHSACKS CHSACSS	CMCTRSMPGK CMCTRSMPGK CWCTFSIPAQ CVCTFSIPAQ	CRCLDIAD-F CRCLDIDD-F CRCLDIDD-F CVCVDMKD-F CVCVDMKD-F	CYKPCES CYKPCES CYEPCK- CYEPCK-
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tr   H6WNA3   tr   Q941H6   tr   A4Q9H3   tr   A4Q9H4				RLNS RLNS RLNS RLNS RLNS	CHSACKS CHSACKS CHSACKS CHSACSS CHSACKS CHSACKS	CMCTREMPGK CMCTREMPGK CMCTREMPGK CVCTESIPAQ CVCTESIPAQ CMCTREMPGK CMCTREMPGK	CRCLDIAD-F CRCLDTDD-F CRCLDTDD-F CVCVDMKD-F CVCVDMKD-F CRCLDTTD-Y CRCLDTTD-F	CYKPCES CYKPCES CYEPCK- CYEPCK- CYKSCKS CYKSCKS
tr   H6WNA3   tr   Q941H6   tr   A4Q9H3   tr   A4Q9H4   tr   A4Q9H4				RLNS RLNS RLNS RLNS RLNS RLNS	CHSACKS CHSACKS CHSACKS CHSACKS CHSACKS CHSACKS CHSACKS	CNCTR SMPGK CNCTR SMPGK CNCTR SMPGK CVCTF SIPAQ CVCTF SIPAQ CNCTR SMPGK CMCTR SMPGK CMCTR SMPGK	CRCLDIAD-F CRCLDTDD-F CRCLDTDD-F CVCVDMKD-F CVCVDMKD-F CRCLDTTD-Y CRCLDTTD-F CRCLDTTD-F	CYRPCES CYRPCES CYEPCK- CYEPCK- CYRSCKS CYRSCKS
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tr   H6WNA3   tr   Q941H6   tr   A4Q9H3   tr   A4Q9H4   tr   A4Q9H4   tr   A4Q976				RLNS RLNS RLNS RLNS RLNS RLNS	CHSACKS CHSACKS CHSACSS CHSACSS CHSACKS CHSACKS CHSACKS CHSACKS	CMCTRSMPGK CMCTRSMPGK CVCTFSIPAQ CVCTFSIPAQ CMCTRSMPGK CMCTRSMPGK CMCTRSMPGK	CRCLDIAD-F CRCLDTDD-F CRCLDTDD-F CVCVDMKD-F CVCVDMKD-F CRCLDTTD-Y CRCLDTTD-Y CRCLDTTD-F CRCLDTTD-F	CYRPCES CYRPCES CYEPCK- CYEPCK- CYEPCK- CYRSCKS CYRSCKS CYRSCKS
tr   H6WNA3  tr   Q941H6  tr   A4Q9H3  tr   A4Q9H3  tr   A4Q9H4  tr   A4Q9H6  tr   A4Q976  tr   C6SX26				RLNS RLNS RLNS RLNS RLNS RLNS RLNS	CHACKS CHACKS CHACKS CHACKS CHACKS CHACKS CHACKS CHACKS CHACKS	CHCTREMPGK CHCTREMPGK CHCTREMPGK CVCTFSIPAQ CVCTFSIPAQ CHCTREMPGK CHCTREMPGK CHCTREMPGK CACTREMPGQ	CRCLDIAD-F CRCLDTDD-F CRCLDTDD-F CVCVDMKD-F CVCVDMKD-F CRCLDTTD-Y CRCLDTTD-Y CRCLDTTD-F CRCLDTTD-F CRCLDTTD-F	CYKPCK: CYKPCE: CYEPCK- CYEPCK- CYKSCK: CYKSCK: CYKSCK: CYKSCK: CYKSCK:
tr   H6MNA3  tr   Q941H6  tr   A4Q9H3  tr   A4Q9H4  tr   A4Q9H4  tr   A4Q976  tr   A6Q976  tr   A0A0R0B				RLNS RLNS RLNS RLNS RLNS RLNS RLNS RLNS	CHACKS CHACKS CHACKS CHACKS CHACKS CHACKS CHACKS CHACKS CHACKS CHACKS	CMCTREMPGR CMCTREMPGR CMCTREMPGR CMCTREMPGR CMCTREMPGR CMCTREMPGR CMCTREMPGR CACTREMPGR CACTREMPGR	CRCLDIAD-F CRCLDTDD-F CRCLDTDD-F CVCVDMKD-F CVCVDMKD-F CRCLDTTD-F CRCLDTTD-F CRCLDTTD-F CRCLDTTD-F CRCLDTTD-F	CYRPCR: CYRPCE: CYEPCK- CYEPCK- CYESCK: CYESCK: CYESCK: CYESCK: CYEPCK-
tr  H6NNA3  tr  Q941H6  tr  A4Q9H3  tr  A4Q9H4  tr  A4Q976  tr  A4Q976  tr  C6SX26  tr  A0A0B2R				RLNS RLNS RLNS RLNS RLNS RLNS RLNS RLNS		CHCTRENPGR CHCTRENPGR CVCTFSIPAQ CVCTFSIPAQ CMCTRENPGR CMCTRENPGR CMCTRENPGR CMCTRENPGR CMCTRENPGQ CACTRENPGQ CACTRENPGQ	CRCLDIAD-F CRCLDTDD-F CRCLDTDD-F CVCVDMKD-F CVCVDMKD-F CRCLDTTD-Y CRCLDTTD-F CRCLDTTD-F CRCLDTTD-F CRCLDTTD-F	CYRPCR: CYRPCE: CYEPCK- CYEPCK- CYRSCK: CYRSCK: CYRSCK: CYRSCK: CYRSCK: CYRSCK: CYRPCK:
tr   46MNA3  tr   2941H6  tr   A4Q9H4  tr   A4Q9H4  tr   A4Q9H4  tr   A4Q976  tr   A4Q976  tr   C6SX26  tr   C6SX26  tr   Q93WK3				RLNS RLNS RLNS RLNS RLNS RLNS RLNS RLNS RLNS	CKSACKS CKSACKS CKSACKS CKSACKS CKSACKS CKSACKS CKSACKR CKSACKR CKSACKP	CMCTREMPGR CMCTREMPGR CWCTESIPAQ CWCTESIPAQ CMCTREMPGR CMCTREMPGR CMCTREMPGR CMCTREMPGR CMCTREMPGQ CACTREMPGQ CACTREMPGQ CLCTESIPAQ	CRCLDIAD-F CRCLDTDD-F CRCLDTDD-F CVUVDMKD-F CRCLDTD-Y CRCLDTTD-Y CRCLDTTD-F CRCLDTTD-F CRCLDTTD-F CRCLDTTD-F CRCLDTTD-F CVCADTND-F	CYRPCR: CYRPCE: CYEPCK- CYEPCK- CYRSCR: CYRSCR: CYRSCR: CYRSCR: CYRSCR: CYRPCR: CYRPCR: CYRPCR:
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tr  460NA3  tr  2941H6  tr  A409H4  tr  A400H4  tr  A4				RLNS RLNS RLNS RLNS RLNS RLNS RLNS RLNS RLNS RLNS RLNS		CHCTRENPGK CMCTRENPGK CMCTRENPGK CWCTFEIPAQ CMCTRENPGK CMCTRENPGK CMCTRENPGK CACTRENPGQ CACTRENPGQ CACTRENPGQ CLCTFEIPAQ CMCTRENPGQ	CRCLDIAD-F CRCLDIDD-F CRCLDIDD-F CVVVDMKD-F CRCLDITD-Y CRCLDITD-Y CRCLDITD-F CRCLDITD-F CRCLDITD-F CRCLDITD-F CRCLDITD-F CRCLDITD-F	CYRPCE: CYRPCE: CYEPCK- CYEPCK- CYRSCK: CYRSCK: CYRSCK: CYRSCK: CYRPCK: CYRPCR: CYEPCK- CYRSCK: CYRSCK:
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Figure 5: Multiple Sequence Alignment of *Cajanus cajan* protease inhibitor (Q5U9N0) with different species. The highly conserved and less conserved residues are highlighted in dark and light grey, respectively. While the conserved cysteine residue as highlighted in yellow

sequence (Uniprot: Q5U9N0) was analyzed and showed that it had 176 amino acid sequences; first 1–19 were signal peptides and rest were chain. The 3D structure created elucidated the presence of 3 beta-sheets, 3 beta-hairpins, 2  $\beta$ -bulges, 6 strands, 3 helices, 1 helix–helix interaction, 41  $\beta$ -turns, and 27  $\gamma$ -turns. To conclude, CCPI crystal can further be refined so that it can be used as a lead molecule in the drug discovery pipeline against tumor cells. With this lead in hand, we intend to exploit it further to study the exact mode of action and elaborated studies in the near future.

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#### **Conflicts of interest**

There are no conflicts of interest.

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