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 targeted along with 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 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 beta-bulges, 6 strands, 3 helices, 1 helix-helix interaction, 41 beta-turns and 27 gamma-turns. **Discussion and Conclusion:** The results indicate that CCPI may help to treat cancer *in vivo* as well. 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 proteasome by either altering, blocking, or preventing access to the active site of enzyme and hence play important role in regulation of proteolysis.[1] 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.[2-5] 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.[6] Furthermore, studies are being performed 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.[7,8] In field of agriculture, to counterbalance the loss caused by chemical pesticides, plant PPIs have gained remarkable attention as natural defense agents in plants.[9] Along with the role of growth inhibition of insects and pests, PPIs also show inhibitory activity of pathogenic nematodes such as *Globodera tabaccum*.[10] and pathogenic fungi such as *Trichoderma reesei*.[11] and *Alternaria alternata*. 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
Plants have the 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. To be specific, pigeon pea (Cajanus cajan L.) seeds contain PIs of trypsin, chymotrypsin, and amylases as well as secondary metabolites and phytoalexins 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 (TI) from C. cajan seeds.

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. 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), Nα-benzoyl-DL-arginine 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 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 μg/ml streptomycin). The cells were cultured at 37°C, 5% CO₂ humid condition in CO₂ 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. 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. CCPI sample was mixed with a sample buffer (0.125-M Tris-HCl pH 6.8, 20% glycerol, 4% SDS, and 10% β-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. The residual enzymatic activity was checked using BAPNA-HCl as substrate. Twenty microliters of trypsin (1 mg/ml) was incubated with 100 μl of CCPI sample and 80 μl of Tris buffer (pH 8.2) for 10 min at room temperature. The reaction was initiated by addition 500 μl 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. 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 × 10⁴ 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:

\[
\text{% cytotoxicity} = \frac{[A]_{\text{control}} - [A]_{\text{test}}}{[A]_{\text{control}}} \times 100
\]

Where \([A]_{\text{control}}\) is the absorbance of control sample and \([A]_{\text{test}}\) is absorbance of test sample.
Screening and developing Cajan 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 ×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. 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 Cajan 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. It also diminished the activity of proteinases of larval midgut showing trypsin-like nature in Manduca sexta. CCPI being smaller in size can be expressed in castor plants to protect them against their lethal pest Achoona janata by inhibiting its midgut trypsin-like proteinases.

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. However, as we know, PIs belonging to BBI family have lower molecular weight of around 6–9 kDa. 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. Further, Osowole et al. isolated PPI from C. cajan weighing ~18.2 kDa. Nioraka et al. showed that CCPIs are only BBI on the basis of gel filtration peaks. Further, Prasad et al. purified BBI-type PI from C. cajan naming it Red Gram PI. Our protocol resulted in purification of CCPI
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 IC₅₀ value of CCPI for A549 cells was ~9.84 µg/ml which was lower as compared to IC₅₀ 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 IC₅₀ values of Lavatera cashmeriana PIs to be 36 ± 2 µg/ml in human lung cancer cell line (NCIH322), which was quite higher than the results obtained in our studies.[4] 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.](image)

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.[5] 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.[6] 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].[7]

![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.](image)

### 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% (NH₄)₃SO₄ precipitate | 3.07                         | 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
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. 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].

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
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 β-bulges, 6 strands, 3 helices, 1 helix–helix interaction, 41 β-turns, and 27 γ-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.

References
1. Koiwa H, Bressan RA, Hasegawa PM. Regulation of protease inhibitors and plant defense. Trends Plant Sci 1997;2:379-84.
2. Sever N, Filipic M, Brzin J, Lah TT. Effect of cysteine proteinase inhibitors on murine B16 melanoma cell invasion in vitro. Biol Chem 2002;383:839-42.
3. Ogawa M, Jing H, Kitto DD, Nakai S, Nakamura S. In vitro anti-cancer activities in Caco-2 and HCT-116 cells of recombinant cystatin C prepared by a Pichia expression system. J Med Food 2003;6:317-22.
4. Heidtmann HH, Salge U, Abrahamson M, Bencina M, Kastelic L, Kopitar-Jeral N, et al. Cathepsin B and cysteine proteinase inhibitors in human lung cancer cell lines. Clin Exp Metastasis 1997;15:368-81.
5. Clawson GA. Protease inhibitors and carcinogenesis: A review. Cancer Invest 1996;14:597-608.
6. Rakashanda S, Qazi AK, Majeeed R, Rafiq S, Dar IM, Masood A, et al. Antiproliferative activity of *Lavatera cresseriana*-protease inhibitors towards human cancer cells. Asian Pac J Cancer Prev 2013;14:3975-8.

7. Asztalos BF, Schaefer EJ, Horvath KV, Cox CE, Skinner S, Gerrior J, et al. Protease inhibitor-based HAART, HDL, and CHD-risk in HIV-infected patients. Atherosclerosis 2006;184:72-7.

8. Greig SL, Deeks ED. Abacavir/dolutegravir/lovirudine single-tablet regimen: A review of its use in HIV infection. Drugs 2015;75:503-14.

9. Farmer EE, Ryan CA. Interplant communication: Airborne methyl jasmonate induces synthesis of proteinase inhibitors in plant leaves. Proc Natl Acad Sci U S A 1990;87:7713-6.

10. Shade RE, Schroeder HE, Puyo JL, Tabo LM, Murdock LL, Higgins TV, et al. Transgenic pea seeds expressing the α-amylase inhibitor of the common bean are resistant to bruchid beetles. Nat Biotechnol 1994;12:793-6.

11. Ishimoto M, Sato T, Chrispeels MJ, Kitamura K. Bruchid resistance oftransgenic azuki bean expressing seed α-amylase inhibitor of common bean. Entomol Exp Appl 1996;79:309-15.

12. Schroeder HE, Gallasch M, Moore A, Tabo LM, Craig S, Hardie DC, et al. Bean α-amylase inhibitor 1 in transgenic peas (*Pisum sativum*) provides complete protection from pea weevil (*Bruchus pisorum*) under field conditions. Plant Physiol 1995;107:1233-9.

13. Haq SK, Atif SM, Khan RH. Protein proteinase inhibitor genes in *Bruchus pisorum*. PLoS One 2015;10:e0124177.

14. Chrispeels MJ, Raikhel NV. Lectins, lectin genes, and their role in plant defense. Ann Rev Phytopathol 1990;28:425-49.

15. Shrivastava B, Ghosh AK, Sankaranarayanan R, Das AK. Crystallization and preliminary X-ray diffraction analysis of a protease inhibitor from the haemolymph of the Indian tasar silkworm, *Antheraea mylitta*. Acta Crystallogr Sect F Struc Biol Cryst Commun 2006;62(Pt 7):669-71.

16. Hamdouai A, Wataleb S, Devreese B, Chiou SJ, Vanden Broeck J, Hamdaoui A, Wataleb S, Devreese B, Chiou SJ, Vanden Broeck J, et al. Purification and characterization of a group of protease inhibitors from *T. castaneum* head of bacteriophage T4. Nature 1970;227:680-5.

17. Roy S, Aravind P, Madhurantakam C, Ghosh AK, Sankaranarayanan R, Das AK. Crystallization and preliminary X-ray diffraction analysis of a protease inhibitor from the haemolymph of the Indian tasar silkworm *Antheraea mylitta*. Acta Crystallogr Sect F Struc Biol Cryst Commun 2006;62(Pt 7):669-71.

18. Haq SK, Atif SM, Khan RH. Biochemical characterization, stability studies and N-terminal sequence of a bi-functional inhibitor from *Phaseolus aureus* Roxb. (Mung bean). Biochimie 2005;87:1127-36.

19. Patil DN, Datta M, Chaudhary A, Tomar S, Sharma AK, Kumar P. Isolation, purification, crystallization and preliminary crystallographic studies of chitinase from tamarind (*Tamarindus indica*) seeds. Acta Crystallogr Sect F Struc Biol Cryst Commun 2009;65(Pt 4):343-5.

20. Chrispeels MJ, Raikhel NV. Lectins, lectin genes, and their role in plant defense. Plant Cell 1991;3:1-9.

21. Garcia-Olmedo G, Duran L, Alba L, Cano M, Bressani R. The nutritional role of polyphenols in beans. In: Bressani R, Elias LG, editors. The nutritional role of polyphenols in beans. Elsevier: 1970:227-680.

22. Roy A, Kucukural A, Zhang Y. TASSER: A unified platform for automated protein structure and function prediction. Nat Protoc 2010;5:725-38.

23. Clementea A, Domenyo C. Biological significance of polymorphism in legume proteinase inhibitors from the Bowman-Birk family. Curr Protein Pept Sci 2006;7:201-16.

24. Imtaiyaz Hassan M, Shajee B, Waheed A, Ahmad F, Sly WS. Carbohydrate binding activity of *Phaseolus vulgaris* lectin. Carbohydr Res 2010;345:107-14.

25. Norioka N, Hara S, Ikenaka T, Abe J. Distribution of the Kunitz and the Bowman-Birk proteinase inhibitors from red gram (*Cajanus cajan*) seeds. J Agric Food Chem 1994;42:68-93.

26. Asztalos BF, Schaefer EJ, Horvath KV, Vladimr VM, Chrispeels MJ, Raikhel NV. Lectins, lectin genes, and their role in plant defense. Cell 1994;61:9-17.

27. Bressani R, Elías LG, Karam G, Karam P, Isolation, purification, crystallization and preliminary crystallographic studies of chitinase from tamarind (*Tamarindus indica*) seeds. Acta Crystallogr Sect F Struc Biol Cryst Commun 2009;65(Pt 4):343-5.

28. Carpenter KJ, Graul AH, March CC, Wortman DR. Insecticidal activity of *I. corrugatus* alpha-amylase inhibitor of common bean. J Insect Sci 2013;13:51.

29. Farmer EE, Ryan CA, Hardie DC, Sadowski TR, Bressani R. The nutritional role of polyphenols in beans. In: Bressani R, Elias LG, editors. The nutritional role of polyphenols in beans. Elsevier: 1970:227-680.

30. Farmer EE, Ryan CA, Hardie DC, Sadowski TR, Bressani R. The nutritional role of polyphenols in beans. In: Bressani R, Elias LG, editors. The nutritional role of polyphenols in beans. Elsevier: 1970:227-680.

31. Bressani R, Elias LG, Karam G, Karam P, Isolation, purification, crystallization and preliminary crystallographic studies of chitinase from tamarind (*Tamarindus indica*) seeds. Acta Crystallogr Sect F Struc Biol Cryst Commun 2009;65(Pt 4):343-5.

32. Carpenter KJ, Graul AH, March CC, Wortman DR. Insecticidal activity of *I. corrugatus* alpha-amylase inhibitor of common bean. J Insect Sci 2013;13:51.

33. Imtaiyaz Hassan M, Shajee B, Waheed A, Ahmad F, Sly WS. Carbohydrate binding activity of *Phaseolus vulgaris* lectin. Carbohydr Res 2010;345:107-14.

34. Bressani R, Elías LG, Karam G, Karam P, Isolation, purification, crystallization and preliminary crystallographic studies of chitinase from tamarind (*Tamarindus indica*) seeds. Acta Crystallogr Sect F Struc Biol Cryst Commun 2009;65(Pt 4):343-5.

35. Carpenter KJ, Graul AH, March CC, Wortman DR. Insecticidal activity of *I. corrugatus* alpha-amylase inhibitor of common bean. J Insect Sci 2013;13:51.

36. Farmer EE, Ryan CA, Hardie DC, Sadowski TR, Bressani R. The nutritional role of polyphenols in beans. In: Bressani R, Elias LG, editors. The nutritional role of polyphenols in beans. Elsevier: 1970:227-680.

37. Carpenter KJ, Graul AH, March CC, Wortman DR. Insecticidal activity of *I. corrugatus* alpha-amylase inhibitor of common bean. J Insect Sci 2013;13:51.

38. Carpenter KJ, Graul AH, March CC, Wortman DR. Insecticidal activity of *I. corrugatus* alpha-amylase inhibitor of common bean. J Insect Sci 2013;13:51.

39. Carpenter KJ, Graul AH, March CC, Wortman DR. Insecticidal activity of *I. corrugatus* alpha-amylase inhibitor of common bean. J Insect Sci 2013;13:51.

40. Carpenter KJ, Graul AH, March CC, Wortman DR. Insecticidal activity of *I. corrugatus* alpha-amylase inhibitor of common bean. J Insect Sci 2013;13:51.

41. Carpenter KJ, Graul AH, March CC, Wortman DR. Insecticidal activity of *I. corrugatus* alpha-amylase inhibitor of common bean. J Insect Sci 2013;13:51.

42. Carpenter KJ, Graul AH, March CC, Wortman DR. Insecticidal activity of *I. corrugatus* alpha-amylase inhibitor of common bean. J Insect Sci 2013;13:51.

43. Carpenter KJ, Graul AH, March CC, Wortman DR. Insecticidal activity of *I. corrugatus* alpha-amylase inhibitor of common bean. J Insect Sci 2013;13:51.

44. Carpenter KJ, Graul AH, March CC, Wortman DR. Insecticidal activity of *I. corrugatus* alpha-amylase inhibitor of common bean. J Insect Sci 2013;13:51.

45. Carpenter KJ, Graul AH, March CC, Wortman DR. Insecticidal activity of *I. corrugatus* alpha-amylase inhibitor of common bean. J Insect Sci 2013;13:51.

46. Carpenter KJ, Graul AH, March CC, Wortman DR. Insecticidal activity of *I. corrugatus* alpha-amylase inhibitor of common bean. J Insect Sci 2013;13:51.