# STUDIES ON BIOLOGICAL ACTIVITY OF BOWMAN-BIRK TRYPSIN INHIBITOR FROM A LOCAL BEAN CULTIVAR

*Thesis*

**by**

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**(H-2015-68-M)**

**Submitted to**


**Dr. YASHWANT SINGH PARMAR UNIVERSITY**
**OF HORTICULTURE AND FORESTRY**
**SOLAN (NAUNI) HP- 173 230 INDIA**

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**Partial fulfillment of the requirements for the degree**

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**DEPARTMENT OF BIOTECHNOLOGY**
**COLLEGE OF HORTICULTURE**

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# CERTIFICATE-I

This is to certify that the thesis titled, **"Studies on biological activity of Bowman-Birk trypsin inhibitor from a local bean cultivar",** submitted in partial fulfillment of the requirements for the award of degree of **MASTER OF SCIENCE MOLECULAR BIOLOGY & BIOTECHNOLOGY** to Dr. Yashwant Singh Parmar University of Horticulture and Forestry, (Nauni) Solan (HP) - 173230 is a bonafide research work carried out by **Ms. Yamini Thakur (H-2015-68-M)** daughter of Shri Yuv Raj under my supervision and that no part of this thesis has been submitted for any other degree or diploma.

The assistance and help received during the course of investigations has been fully acknowledged.

**Place : Nauni-Solan**
**Dated:**

**Dr (Mrs.) Amarjit K Nath**
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# CERTIFICATE-II

This is to certify that the thesis titled, **"Studies on biological activity of Bowman-Birk trypsin inhibitor from a local bean cultivar"** submitted by **Ms. Yamini Thakur (H-2015-68-M)** daughter of Sh. Yuv Raj to Dr Yashwant Singh Parmar University of Horticulture and Forestry, Nauni, Solan (HP) in partial fulfilment of the requirements for the award of degree of **MASTER OF SCIENCE MOLECULAR BIOLOGY & BIOTECHNOLOGY** has been approved by the Student's Advisory Committee after an oral examination of the same in collaboration with the external examiner.

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This is to certify that all the mistakes and errors pointed out by the external examiner have been incorporated in the thesis titled, **"Studies on biological activity of Bowman-Birk trypsin inhibitor from a local bean cultivar",** submitted by **Ms. Yamini Thakur (H-2015-68-M)** daughter of shri Yuv Raj to Dr Yashwant Singh Parmar University of Horticulture and Forestry, Nauni, Solan (HP) in partial fulfillment of the requirements for the award of degree of **MASTER OF SCIENCE MOLECULAR BIOLOGY AND BIOTECHNOLOGY.**

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**Place: Nauni, Solan**
**Date:** **( YAMINI THAKUR )**

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# ABBREVIATION

| | |
|---|---|
| % | Per cent |
| °C | Degree centigrade |
| µg | Microgram |
| µl | Microlitre |
| APS | Ammonium per sulphate |
| BapNA | N-Benzoylarginine p-nitroanilide |
| $CaCl_2$ | Calcium chloride |
| Cm | Centimeter |
| CRD | Completely randomized design |
| $CuSO_4$ | Copper sulphate |
| g | Gram |
| M | Molar |
| mA | Milliampere |
| mg | Milligram |
| ml | Milliliter |
| mM | Millimolar |
| mm | Millimeter |
| N | Normal |
| $Na_2CO_3$ | Sodium carbonate |
| $Na_2HPO_4$ | Sodium hydrogen phosphate |
| NaCl | Sodium chloride |
| $NaH_2PO_4$ | Sodium dihydrogen phosphate |
| $NaKC_4H_4O_6.4H_2O$ | Sodium potassium tartarate |
| NaOH | Sodium hydroxide |
| nm | Nanometer |
| OD | Optical density |
| rpm | Revolutions per minute |
| SDS | Sodium dodecyl sulphate |
| TIA | Trypsin inhibitor activity |
| TUI | Trypsin units inhibited |
| viz. | Namely |
| w/v | Weight by volume |

# *Chapter-1*

# INTRODUCTION

Protease inhibitors in plant play essential roles in biological systems, regulating proteolytic processes and participate in defense mechanisms against attack by a large number of insects, fungi and other pathogenic microorganisms (Lopes *et al*., 2009). Most storage organs such as seed and tuber contains 1-10% of total protein as protease inhibitors. On the basis of specificity towards proteolytic enzymes, protease inhibitors are classified as serine, cysteine, aspartic and metallo-proteinase inhibitors (Mourao and Schwartz, 2013). Out of these, most extensively studied protease inhibitors are serine protease inhibitors, found in leguminosae family (Macedo *et al*., 2011; Prasad *et al*., 2010). These protease inhibitors are small, stable and abundant proteins showing specificity towards trypsin and chymotrypsin (Bode and Huber, 2000). Leguminous plant seeds usually contain two major types of serine protease inhibitors, Bowman-Birk inhibitor (8-16 kDa) with seven disulfide linkages, high cysteine content, greater stability to pH and have two reactive sites for trypsin and chymotrypsin and the second one is Kunitz type (20-25 kDa) having two disulfide linkages, low cysteine content and a single reactive sites for trypsin (Laskowski and Qasim, 2000)

Pathogenic fungi can be hazardous for humans and plants (Gauthier and Keller, 2013). They may be a cause of infection in humans and when uncontrolled could be fatal. In plants, fungal infection can kill the plants and causing losses of agricultural commodities in many zones of the world. These losses can occur on growing in-field crops as well as harvested commodities, leading to damage ranging from rancidity, odour, flavour changes, loss of nutrients, and germ layer destruction. Crop losses due to pathogens are often more severe in developing countries (e.g. cereals, 22%) as compared to those in developed countries (e.g. cereals, 6%) (Oerke *et al*., 1994). For individual crops, Oerke and Dehne (2004) estimated that worldwide, fungal losses can be 100% if a susceptible cultivar is planted or the climate is favorable in any year, and down to 0%, if resistant varieties are planted, fungicides used and good husbandry employed. *Aspergillus*, *Alternaria* and *Fusarium* are amongst the most common fungal species associated with growth and damage to food crops in the field.

Fungal hyphae secrete proteases to enter the plant cells and then absorb nutrients from the leaves. Protease inhibitors act against fungal proteases and enhanced resistance against pathogenic fungi. Protease-deficient fungal mutants failed to produce lesions in plants. Inhibitors of serine (e.g., trypsin and chymotrypsin) and cysteine proteases potently act against fungi which are pathogenic to plants and animals (Joshi *et al*., 1998). To date, antifungal peptides found in common bean have been reported to be defensins. They are a diverse group of low molecular mass cysteine rich proteins found in mammals, fungi, insects and plants (Wong *et al.*, 2007). Many phytopathogenic bacteria are known to produce extracellular proteinases (Kalashnikova *et al*., 2003) which may play an active role in the development of diseases. In response to such attack by proteinases, plants synthesize inhibitory polypeptides that can suppress the enzyme activities. Hence protease inhibitors from plants potently inhibit the growth of a variety of pathogenic bacterial and fungal strains and are therefore excellent candidate for the development of antimicrobial agent (Tian and Zhang, 2005; Shulke and Murdock, 1983).

Cancer has been recognized as a public health problem. The 57% (8 million) of new cancer cases, 65% (3.3 million) of the cancer deaths and 48% (15.6 million) of the five years prevalent cancer cases were found in the less developed world region (Ferlay *et al*., 2015). So far, crops such as beans, potatoes, barley, squash, millet, wheat, buckwheat, groundnut, chickpea, pigeonpea, corn, and pineapple have been identified as good sources of protease inhibitors. Protease inhibitors in plants make them incredible sources to determine novel PIs with specific pharmacological and therapeutic effects due to their peculiarity and superabundance. The consumption of common bean as part of the diet has been related to a reduction of the incidence of colorectal cancer (Lanza *et al*., 2006).

Several plant PIs are under further evaluation in *in vitro* clinical trials. Recently it has been suggested that the Kunitz trypsin inhibitor (KTI) and Bowman-Birk inhibitor (BBI) suppresses both initiation and promotion stages of carcinogenesis (Kennedy, 1993). In particular, Bowman-Birk appear to be highly promising as a cancer chemo-preventive agent. Consequently, several methods for making Bowman-Birk inhibitor have been suggested by Sessa and Wolf (2001).

The research on common bean protease inhibitors not only focuses on their potential as biological control agent for phytopathogenic fungi and bacteria but it also explores their human health benefits such as antioxidants and anti-cancer potential (Ferlay *et al*., 2015).

Plant protease inhibitors have been purified and characterized from a wide variety of legume seeds such as *Vicia faba* (Fang *et al.*, 2010), *Phaseolus vulgaris* (Chan *et al*., 2013), *Cicer arietinum* L. (Yili *et al*., 2015) *Inga vera* (Bezerra *et al*., 2016). Hence it is highly desirable to identify and characterize novel protease inhibitors for their multi-purpose values from plant resources. Keeping in view the above facts the present studies will be undertaken with following objectives:

i) Partial purification of Bowman-Birk trypsin inhibitor from local bean cultivar.

ii) Measurement of its biological activity.

# *Chapter-2*

# REVIEW OF LITERATURE

Relevant literature pertaining to present study entitled as **"Studies on biological activity of Bowman-Birk trypsin inhibitor from a local bean cultivar"** has been reviewed under the following main headings:



## 2.1 PARTIAL PURIFICATION OF TRYPSIN INHIBITOR

Purification of protease is important for studying the properties and for better understanding of the functioning of the enzyme. Majority of the procedures involve concentration of the seed extract either using salt or solvent precipitation or by ultrafiltration. The concentrated enzyme is then subjected to variety of chromatographic procedures such as ion exchange chromatography (cationic or anionic using CM-cellulose, DEAE-cellulose, phenyl-sepaharose), affinity chromatography (using matrix bound to inhibitor or substrate or CNBr-activated sepharose) and gel filtration chromatography (using sephadex or Biogel matrix). Apart from these conventional procedures, others such as fast protein liquid chromatography (FPLC) and preparative PAGE (Wasko *et al.*, 2012) have also been used for the purification of the proteases. Many times more than one method is required to obtain homogeneous preparation.

Benjakul *et al.* (2000) isolated trypsin inhibitor from cultivars of cowpea, pigeonpea and bambara groundnuts which are grown in Thailand. They reported that extraction of seeds with NaCl reduced the recovery of trypsin inhibitor as compared to other solvents and also found that three hours of extraction time was optimum for the recovery of trypsin inhibitor from pigeonpea and bambara groundnuts. Whereas, one hour was optimum for cowpea. The partial purification of inhibitors accomplished by heat treatment at 90°C for 10 minutes followed by ammonium sulfate precipitation with 30-65% saturation. The partially purified inhibitors were heat stable up to 30 minutes at 90°C. The activities were retained over a wide

range of pH at 25°C but were lost when samples were treated with β-mercaptoethanol prior to electrophoresis.

Deshimaru *et al*. (2002) purified proteinase inhibitors, I-VIIa, VIIb, and VIII, from wild soya seeds by ammonium sulfate fractionation and successive chromatographies on SP-Toyopearl 650M, Sephacryl S-200SF and DEAE-Toyopearl 650S columns. Reverse-phase HPLC gave pure inhibitors. They classified two inhibitors (VIIb and VIII) with a molecular weight of 20,000 daltons as a soybean Kunitz inhibitor and others (I-VIIa) with a 8,000 daltons molecular weight were classified as Bowman-Birk inhibitor.

Kumar *et al.* (2002) purified three Bowman-Birk inhibitors (HGGI-I, II and III) from cotyledons of germinated horsegram seeds (*Dolichos biflorus* L.) by size-exclusion chromatography and ion-exchange chromatography. They found these inhibitors to differ from each other in amino acid composition, molecular size, charge and amino-terminus ends. The amino acid sequence of HGGI-II was found to be identical with HGGI-I except for the loss of a single amino-terminal aspartyl residue. Whereas, HGGI-III was found to show loss of a pentapeptide. They reported these three inhibitors to be potent competitive inhibitors of trypsin and chymotrypsin.

Sammour (2005) isolated four isoinhibitors of trypsin from *Vigna unguiculata* (L.) walp. They purified crude protein by 50-70 % ammonium sulphate saturation, DE-52 cellulose column (pH 8) and Sephacryl S-200. On SDS-PAGE, the active material recovered from the column showed only one band, which was designated as protease isoinhibitor IB (CPTI IB), II (CPTI II) and III (CPTI III).

Wang *et al.* (2006) isolated novel protease inhibitor from mung bean, designated mungoin, having molecular mass of 10 kDa as determined by SDS-PAGE. The isolation procedure involved a combination of extraction, ammonium sulfate precipitation, ion exchange chromatography on CM-Sephadex, and high-performance liquid chromatography (HPLC) on SP-Toyopearl. Its isoelectric point was found to be 9.8. Its N-terminal amino acid sequence was EMPGKPACLDTDDFCYKP, demonstrated some resemblance to the C-terminal sequences of other protease inhibitors and inhibitor precursors from leguminous plants.

Mirjana *et al.* (2007) studied two major trypsin inhibitors (TI) in soybean, i.e., the Kunitz (KTI) and Bowman-Birk (BBI) trypsin inhibitors. Twelve soybean genotypes were investigated and they showed significant differences in TIA. Highest TIA was detected in Krajina, 100.95 TUI/mg genotype while lowest TIA in Lana 60.36 TUI/mg.

Chaudhary *et al.* (2008) purified a highly stable and potent trypsin inhibitor to homogeneity from the seeds of *Putranjiva roxburghii* by acid precipitation, cation-exchange and anion-exchange chromatography. Protein was reported to consists of a single polypeptide chain with molecular mass of approximately 34 kDa, analysed by SDS-PAGE. The purified inhibitor inhibited bovine trypsin in 1:1 molar ratio. The inhibitor retained the inhibitory activity over a broad range of pH (pH 2-12), temperature (20-80°C) and in presence of DTT (up to100 mM). Two peptides obtained by internal partial sequencing showed significant resemblance to Kunitz-type inhibitors.

Guillamon *et al.* (2008) studied trypsin inhibitors in grain legume seeds from different species and cultivars. They reported trypsin inhibition content to range from negligible in *Lupinus* spp. to very high in *Glycine max*. The highest TIU $mg^{-1}$ was in soybean (43–84) and common bean (21–25). Inhibitor content of different *Lathyrus* cultivars, ranged from 19-30 TIU $mg^{-1}$ sample. It was higher than the contents in chickpea (15–19 TIU $mg^{-1}$ sample) and pea (6–15 TIU $mg^{-1}$ sample). Lentil and faba bean had low values in most cultivars (3–8 and 5–10 TIU $mg^{-1}$ sample, respectively). Trypsin inhibitor isoform analysis showed the amount of TI to vary with legume species and variety.

Scarafoni *et al.* (2008) purified trypsin inhibitor from *Lupinus albus* L. The protein was isolated and characterized by direct amino acid sequencing, MALDI-TOF mass spectroscopy and circular dichroism. The inhibitor had 63 amino acid residues, the molecular weight was found to be 6.8KDa and it had isoelectric point of 8.2. It inhibited two trypsin molecules simultaneously but not chymotrypsin. BLAST search against UniProtKB/TrEMBL database indicated the inhibitor to belong to Bowman-Birk inhibitor (BBI) family.

Ying (2009) isolated and purified trypsin inhibitor (PVTI) from *Phaseolus vulgaris* by ion exchange and gel filtration chromatography. SDS-PAGE indicated three protein bands of molecular weight 34, 16 and 15 kD respectively. The purified enzyme on isoelectric focusing (IEF) had pI values of 5.25 and they reported PVTI to show irreversible inhibiton.

Prasad *et al.* (2010) purified a proteinase inhibitor (BgPI) from black gram, *Vigna mungo* (cv. TAU-1) seeds by ammonium sulfate fractionation, followed by ion-exchange, affinity and gel-filtration chromatography. BgPI showed a single band in SDS–PAGE with an apparent molecular mass of □ 8 kDa. BgPI existed in different isoinhibitor forms with pI values ranging from 4.3 to 6.0. The internal sequence ‘‘SIPPQCHCADIR” of a peak 1453.7 m/z, obtained from MALDI-TOF showed 100% similarity with Bowman-Birk inhibitor (BBI) family. BgPI exhibited non-competitive type inhibitory activity against both bovine pancreatic trypsin (Ki of 309.8 nM) and chymotrypsin (Ki of 10.7 lM), with a molar ratio of 1:2 with trypsin. Lysine residue(s) present in the reactive site of BgPI played an important role in inhibiting the bovine trypsin activity. BgPI was stable up to a temperature of 80˚C and active over a wide pH range between 2 and 12.

Klomklao *et al*. (2011) extracted, trypsin inhibitor from mung bean (*Vigna radiata* (L.) R. Wilczek) seeds. They attained optimal extraction by shaking the defatted mung been seed powder in distilled water ($P < 0.05$). The extraction time affected the inhibitor recovery significantly ($P < 0.05$). They showed that extraction time of 2 h was optimum for the recovery of the trypsin inhibitor. They purified trypsin inhibitor from mung bean by heat-treatment at 90°C for 10 min, followed by ammonium sulphate precipitation with 30–65% saturation and gel filtration on Sephadex G-50. Molecular weight distribution and inhibitory activity staining showed the purified trypsin inhibitor to have a molecular weight of 14 kDa. They found purified inhibitor to be heat stable up to 50 min at 90°C. The inhibitory activity was retained over a wide pH range.

Sharma and Suresh (2011) purified a Kunitz-type trypsin inhibitor protein (CPTI) from chickpea seeds. They estimated molecular mass of the inhibitor to be 18,000 daltons on SDS-PAGE. The inhibitory activity of purified CPTI was 114 TIU (trypsin inhibitory units) per milligram of protein, which was high as compared to other known Kunitz-type trypsin inhibitors from legumes.

Satheesh and Murugan (2012) reported 14.4 kDa *Coccinia grandis* protease inhibitor (CGPI) from leaves of *Coccinia grandis* and purified it to homogeneity by ammonium sulfate precipitation, Sephadex G-75 column, DEAE Sepharose column and trypsin-Sepharose affinity chromatography. They estimated molecular mass by size exclusion chromatography and results obtained agreed with SDS-PAGE. They found that CGPI to exhibit remarkable stability at temperature of upto 80°C and over a wide range

of pH (2-12). It completely lost its inhibitory activity against trypsin and chymotrypsin when incubated with DTT. Lysine residue present in the active site of CGPI are essential for inhibiting the trypsin activity.

Chan *et al.* (2013) purified trypsin inhibitor from the seeds of *Phaseolus vulgaris* cv. brown kidney bean with a molecular mass around 17 kDa. Their purification protocol involved, affinity chromatography on Affi-gel blue gel, ion-exchange chromatography on Q-Sepharose and Mono Q, and gel filtration on Sephadex G-75. The molecular size and N-terminal amino acid sequence of the trypsin inhibitor resembled leguminous Bowman-Birk protease inhibitors (BBIs), signifying that brown kidney bean trypsin inhibitor is BBI, it showed trypsin inhibitory activity at all pH values (0-14) and up to 90°C.

Chandrashekharaiah (2013) purified trypsin inhibitor from seeds of *Mucuna pruriens* employing ammonium sulfate fractionation, cation exchange chromatography on CM-cellulose and gel-permeation chromatography on Sephadex G-100. The purified *Mucuna pruriens* trypsin inhibitor (MPTI) showed a specific inhibitor activity of 474.66, fold purity of 99.51 and the yield obtained was 22.08%. The homogeneity of the purified preparation was confirmed by PAGE, IEF and SDS–PAGE. The molecular weight determined by gel-permeation chromatography and SDS–PAGE were 12 and 11.6 kDa respectively. MPTI was found to be stable at all temperatures between 0 and 90 ºC and pH 2.0 – 10 and hence exhibited high stability.

Chan *et al.* (2014) purified 16-kDa trypsin inhibitor from small pinto bean by anion exchange and size chromatography Q-Sepharose, Mono Q and Superdex 75 columns. Small pinto bean trypsin inhibitor demonstrated moderate pH stability (pH 2–10) and marked heat stability, with its trypsin inhibitory activity largely retained after exposure to 100°C for half an hour. The activity was abolished in the presence of dithiothreitol, in a dose-dependent manner, implying that disulfide bonds in small pinto bean trypsin inhibitor are crucial for the activity.

Gu *et al.* (2014) isolated Bowman-Birk trypsin inhibitor from soybean by a combination of alcohol precipitation, thermal denaturation, isoelectric precipitation and acetone precipitation. The extract was purified by DE-52 ion exchange and affinity chromatography to 50.07-fold with trypsin activity of 822.31 U $mg^{-1}$. The purified SBBI gave a single protein band in SDS-PAGE electrophoresis. The accurate molecular mass of this

inhibitor was 8837.46 Da by MALDI-TOF. N-terminal sequence showed high homology with other serine proteinase inhibitors belonging to the *Leguminosae* family.

Kuhar *et al*. (2014) purified a double headed PI, active against both trypsin and chymotrypsin, from *Dolichos biflorus* to 14 fold with 84% recovery using an immobilized metal affinity chromatography (IMAC) medium consisting of Zn- alginate beads. The purified inhibitor protein showed a single band on SDS-PAGE corresponding to molecular mass of 16kDa and was stable over a pH range of 2.0-20 and upto temperature of 100°C for 20 minutes. They observed optimum temperature for trypsin and chymotrypsin inhibitor to be 50°C and 37°C, respectively and optimum pH was 7.0 and 8.0, respectively.

Mojica and Mejia (2015) prepared bean protein isolates (BPI) from 15 common bean cultivars and hydrolyzed then using pepsin/pancreatin. Thirteen proteins were identified by SDS-PAGE and proteinin-gel tryptic-digestion-LC/MS. Protein profile was similar among common bean cultivars with high concentrations of defense-related proteins. Major identified proteins were phaseolin, lectin, protease and α-amylase inhibitors. Lectin(159.2 to 357.9 mg lectin/g BPI), Kunitz trypsin inhibitor(inh) (4.3 to 75.5 mg trypsin inh/g BPI), Bowman-Birk inhibitor (5.4 to 14.3 µg trypsin-chymotrypsin inh/g BPI) and α-amylase inhibitor activity (2.5 to 14.9 % inhibition relative toacarbose/mg BPI) were higher in Mexican beans compared to Brazilian beans. Abundant peptides were identified by HPLC MS/MS with molecular masses ranging from 300 to 1500 Da and significant sequences were SGAM, DSSG, LLAH,YVAT, EPTE and KPKL. Peptides from common bean proteins presented potential biological activities.

Pesoti *et al.* (2015) purified a novel trypsin inhibitor of protease (CqTI) from *Chenopodium quinoa* seeds. They used optimal extracting solvent 0.1M NaCl pH 6.8 ($p < 0.05$). They reported that extraction time of 5 hours and 90°C optimum for the recovery of trypsin inhibitor from *C. quinoa* seeds. The purification was carried out by gel-filtration and reverse phase chromatography. Purified CqTI was active against commercial bovine trypsin and chymotrypsin and had a specific activity of 5,033.00 (TIU/mg), which was purified to 333.5 fold. The extent of purification was determined by SDS-PAGE. CqTI had an apparent molecular weight of approximately 12 kDa and two bands in reduced conditions as determined by Tricine-SDS-PAGE. MALDI-TOF showed two peaks in 4,246.5 and 7,908.18m/z. CqTI showed high levels of essential amino acids. N-terminal amino acid sequence of this protein did not show similarity to any known protease inhibitor. Its activity

was stable over a pH range (2.0-12.0), temperatures range (20-100°C) and in presence of reducing agents.

Thakur *et al.* (2015) analysed nine *Phaseolus vulgaris* L. cultivars of Himalayan region for trypsin and α-amylase inhibitor activity. They reported maximum trypsin inhibitor activity (TUI) per gram seed flour in Baspa cultivar (3100.833±2.674) and minimum in Luxmi cultivar (1026.250±4.070), while the total soluble protein (mg per gram seed flour) was maximum in Capsule cultivar (154.984±0.384) and minimum in Contender cultivar (105.034±0.408).

Yili *et al.* (2015) extracted and purified proteins fraction of chickpea sprout by two steps of ion-exchange chromatography and high-pressure liquid chromatography (HPLC) for the first time, the peptides components and molecular weights were found to be 11079.57, 11440.95, 16619.09, 26016.41, 26032.49, 26016.34 and 28822.02 Da by liquid chromatography and mass spectrometer (LC/MS). They isolated a major protein component of this 26032.49 kDa fraction with trypsin inhibitor activity by HPLC and the partial amino acid sequence was determined as the:

(K)LIEAMVEVEGQLCMDVPSNPGTSAPPFAIVHSSGISLPDRQSATPCSAD-DWRPYLV(-).

Dabhade *et al.* (2016) extracted a novel, thermostable, non-toxic, proteinaceous trypsin inhibitor from seeds of *Albizia amara* Boiv. They purified protease inhibitor (API) by acetone fractionation, ion-exchange chromatography (diethy-aminoethyl (DEAE) cellulose), and gel permeation chromatography (GPC; Sephadex G-100). The molecular weight of API was found to be 49 kDa and it was identified as a serine protease inhibitor. The API remained active over a wide pH range (3.0–8.0), and showed thermal stability at 60°C. They reported API to retain 85% trypsin inhibition activity upon storage at 4◦C over a period of 6 months.

Shah *et al.* (2016) isolated, purified and characterized a protein of about 20 kDa, named PotHg, showing hemagglutination activity from tubers of Indian potato, *Solanum tuberosum*. The sequencing and MS/MS analysis confirmed that the purified protein to be a Kunitz-type serine protease inhibitor having two chains (15 kDa and 5 kDa). SDS and native PAGE analysis showed that the protein was glycosylated and was a heterodimer of about 15 kDa subunits. PotHg retained hemagglutination activity over a pH range 4-9 and up to 80°C.

Mannose and galactose interacted with the PotHg with a dissociation constant (Kd) of 1.5 × $10^{-3}$ M and 2.8 × $10^{-3}$ M. Circular dichroism (CD) studies showed PotHg to contain mostly sheets (~45%) and loops which is in line with previously characterized protease inhibitors and modeling studies. There are previous reports of Kunitz-type protease inhibitors showing lectin like activity from *Peltophorum dubium* and *Labramia bojeri*. This is the first report of a Kunitz-type protease inhibitor showing lectin like activity from a major crop plant.

Coscueta *et al.* (2017) used an analytical method, which involves a continuous spectrophotometric rate determination for trypsin activity against the substrate N-benzoyl-DL-arginine p-nitroanilide, as an alternative to the standard discontinuous assay. They analysed that stopping the reaction with acetic acid and a centrifugation step to decrease turbidity are not required, thus reducing costs and sample preparation time. The TI activity of different flour samples, determined by both assays, demonstrated to be statistically comparable, irrespective of the TI concentration level. The coefficients of variation of the novel method did not exceed 8% at any concentration level. The curves of progress reaction showed a non-linear behavior in samples without TI. A reduction of incubation time from 10 min to 2 min increased the method sensitivity and extended its linear range. A more economical, faster and simpler assay was developed.

Li *et al.* (2017) purified 17.5-kDa trypsin inhibitor from *Phaseolus vulgaris* cv. “gold bean” by ion exchange chromatography on DEAE-cellulose (Diethyl amino ethyl cellulose), affinity chromatography on Affi-gel blue gel, ion exchange chromatography on SP-sepharose (Sulfopropyl-sepharose), and gel filtration by FPLC (Fast protein liquid chromatography) on Superdex 75.

## 2.2 BIOLOGICAL ACTIVITY OF TRYPSIN INHIBITOR

### 2.2.1 Antimicrobial activity of trypsin inhibitor

The frequency of life-threatening infections caused by pathogenic microorganisms has increased worldwide and is becoming an important cause of morbidity and mortality in developing countries. The rapid emergence of microbial pathogens that are resistant to currently available antibiotics has triggered considerable interest in the isolation and investigation of the mode of action of antimicrobial proteins (Alasbahi and Melzig, 2008).

Protease inhibitors are ubiquitous in nature and in plants they are abundant in tubers and seeds. These PIs believed to act as storage proteins and as defense mechanism (Bergey *et al*., 1996). Protease inhibitors control the action of proteases that are indispensable for the growth and development of the organism. They play an important role in the protection of plant tissues from pest and pathogen attack (Vernekar *et al*., 2001).

Chilosi *et al.* (2000) reported trypsin inhibitor from wheat kernel (WTI) to have strong antifungal activity against a number of pathogenic fungi. Under lab conditions, WTI inhibited spore germination and hyphal growth of pathogens. Protein concentration required for 50% growth inhibition ($IC_{50}$) ranged from 111.7 to above 500 µg/ml. WTI was reported to cause morphological alterations represented by hyphal growth inhibition and branching. One of the fungal species *Botrytis cinerea* produced a trypsin-like protease, which was inhibited by the trypsin inhibitor.

Ye *et al*. (2001) isolated Bowman-Birk type trypsin-chymotrypsin inhibitor from broad beans. They obtained fractions from broad bean (*Vicia faba*) extract and monitored them for antifungal activity. They found broad bean trypsin-chymotrypsin inhibitor exerted a prominent suppressive action on mycelia growth of *Fusarium oxysporium* and *Mycospharella arachidicola* and a less notable inhibitory effect on growth of *Botrytis cinerea*.

Ng *et al.* (2003) isolated trypsin inhibitor (TI) from seeds of *Clausena lansium* (Lour) Skeels with a very simple procedure. The resulted trypsin inhibitor exerted antifungal activity towards *Physalospora piricola* but not *Mycosphaerella arachidicola*, *Botrytis cinerea*, *Fusarium oxysporum* and *Coprinus comatus*.

Hermosa *et al.* (2006) reported potato (*Solanum tuberosum* var. Desiree) PI to show strong inhibitory activity not only against *Botrytis cinerea* fungal proteases but also towards spore germination, hyphal elongation and development of necrotic lesions.

Wang *et al.* (2006) isolated novel protease inhibitor from mung bean, designated mungoin, with both antifungal and antibacterial activities. It exerted a potent inhibitory action toward a variety of fungal species including *Physalospora piricola, Mycosphaerella arachidicola, Botrytis cinerea, Pythium aphanidermatum, Sclerotium rolfsii* and *Fusarium oxysporum*, as well as an antibacterial action against *Staphylococcus aureus*.

Lopes *et al.* (2009) purified three serine protease inhibitors isoforms, denoted ApTIA, ApTIB, and ApTIC from *Acacia plumosa* Lowe seeds. They reported *Aspergillus niger, Thielaviopsis paradoxa* and *Colletotrichum sp.* to be inhibited by each isoforms. These three potent inhibitors from *A. plumose* may therefore be of great interest as specific inhibitors to regulate proteolytic processes.

Zhang *et al.* (2009) purified trypsin inhibitor from wild type soybean (Glycine soya) and domesticated soybean (Glycine max) on affinity chromatography column. They reported SBTI (domesticated soybean trypsin inhibitor) and WBTI (wild type soybean trypsin inhibitor) to inhibit *Aspergillus flavus* growth. This growth inhibition was possibly the result of inhibition of α-amylase activity of *Aspergillus flavus*.

Fang *et al.* (2010) isolated Bowman Birk type trypsin inhibitor (termed VFTI-E1) from faba beans (*Vicia faba* cv. Egypt 1) and demonstrated antifungal activity toward the filamentous fungus *Valsa mali* with $IC_{50}$ of 20 μM. The mechanism of its antifungal action toward *V. mali* included induction of alteration of hyphal morphology, growth inhibition by chitin deposition at hyphal tips and permeabilization of fungal membrane.

Wang and Rao (2010) purified trypsin-chymotrypsin inhibitor having both antifungal and antibacterial activity from the large lima bean (*Phaseolus limensis*) legumes. They reported these inhibitor protein exerted potent antifungal action toward *Botrytis cinerea, Alternaria alternate (Fr.) Keissl,* and *Pythium aphanidermatum*..

Satheesh and Murugan (2011) reported antimicrobial activity of protease inhibitor (PIs), isolated from *Coccinia grandis* (L.) Voigt. PI strongly inhibited pathogenic microbial strains, including *Staphylococcus aureus, Klebsiella pneumonia, Proteus vulgaris, Eschershia coli, Bacillus subtilis* and pathogenic fungus *candida albicans, Mucor indicus, Penicillium notatum, Aspergillus flavus* and *Cryptococcus neoformans.* Examination by bright field microscopy showed inhibition of mycelial growth and sporulation. Morphologically, PI treated fungus showed a significant shrinkage of hyphal tips.

Rakashanda *et al.* (2012) purified protease inhibitors from the seeds of *Lavatera cashmeriana* Camb and it was screened for antibacterial activity against *Klebsiella pnuemoniae, Escherichia coli* and *Pseudomonas aeruginosa*.

Chandrashekharaiah (2013) purified trypsin inhibitor from seeds of *Mucuna pruriens* and reported its antifungal activity against *Aspergillus niger* and *Trichoderma viridae*. Hence due to its antifungal activity property, they suggested it as potent agent to control unwanted proteolytic processes.

Hong *et al.* (2013) isolated and purified a novel trypsin inhibitor with considerable thermal and pH stability designated *Glytine,* from seeds of the Chinese black soybean *Glycine max* (L.) *Merr*. The 20 N-terminal amino acid sequences were determined to be DEYSKPCCDLCMCTRRCPPQ, demonstrating close homology with the sequences of leguminous trypsin inhibitors. The inhibitory activity of *Glytine* was unaffected by exposure to temperatures up to 100°C, or within the pH range 2-12. The inhibitor showed antifungal activity against *Pythium aphanidermatum, Fusarium oxysporum, Alternaria alternata (Fr.) Keiss, Fusarium solani* and *Botrytis cinerea*.

Nair and Sandhu (2013) purified 20 kDa trypsin inhibitor from Fusarium wilt resistant cultivar chickpea (*Cicer arietinum* L.) (viz. JG 2001-12). They reported Fusarium wilt resistant cultivar to have high trypsin inhibitory activity (99%) in the presence of trypsin enzyme. In their preliminary studies, they used crude extracts of JG 2001- 12 that showed decrease in radial growth of *Fusarium oxysporum* f.sp. *ciceris* (*Foc*). They observed 45% - 82% reduction in conidium germination at 20 $\mu g \cdot mL^{-1}$ *Cicer arietinum* trypsin inhibitor (CaTI) concentration.

Dabhade *et al.* (2016) purified a novel, thermostable, non-toxic, proteinaceous trypsin inhibitor from seeds of *Albizia amara* Boiv. They reported antimicrobial effectiveness of the API against *Pseudomonas aeruginosa, Bacillus subtilis, Alternaria alternata, Alternaria tenuissima,* and *Candida albicans*.

Macedo *et al.* (2016) isolated number of protease inhibitor from plants and particularly from seeds having antimicrobial activity. A 20,000 Da serine peptidase inhibitor, named ILTI, was isolated from *Inga laurina* seeds and showed potent inhibitory enzymatic activity against trypsin. The aim of this study was to determine the effects of ILTI on the growth of pathogenic and non-pathogenic microorganisms. They reported ILTI strongly inhibit the growth of *Candida tropicalis* and *Candida buinensis*. However, it was ineffective against human pathogenic bacteria. They also investigated the potential of ILTI to permeabilize the plasma membrane of yeast cells. *C. tropicalis and C. buinensis* were

incubated for 24 h with the ILTI at different concentrations, which showed that this inhibitor induced changes in the membranes of yeast cells, leading to their permeabilization. In conclusion, results indicated the ability of peptidase inhibitors to induce microbial inhibition; therefore, they might offer templates for the design of new antifungal agents.

### 2.2.3 Anticancerous activity of trypsin inhibitor

Proteases, also known as proteolytic enzymes, are enzymes that catalyze the breakdown of proteins by hydrolysis of peptide bonds. Proteases are extremely important signaling molecules that are involved in numerous vital processes like apoptosis, cell growth and activation, adhesion, invasion, cell migration and metastasis, protein secretion, cellular interactions and signal transduction. Thus, show complete anticancer mechanism.

Proteases have been found to be involved in tumor growth and progression. Inhibitors of such proteases are emerging with promising therapeutic uses in the treatment of cancer. Protease inhibitor suppression of carcinogenesis is related to ability to effect the expression of certain oncogene and the levels of certain types of proteolytic activities. Protease inhibitors being used in anticancer therapy have been isolated from plants, bacteria and prepared synthetically. Different protease inhibitors have been used and are being in clinical and pre-clinical stage. Thus, studying PIs as anticancer agents open a new field for treatment of cancer.

Armstrong *et al*. (2000) identified Bowman-Birk inhibitor (BBI) as a potential cancer chemopreventive agent for humans. In Phase I clinical trial, BBI concentrate was administered as a single oral dose to 24 subjects with oral leukoplakia. Pharmacokinetics of BBI was analyzed, and subjects were monitored clinically for toxic effects. Subjects received between 25 and 800 chymotrypsin inhibitor units (CIU) of the compound in a dose escalation trial. BBI was taken up rapidly, and a metabolic product of BBI was excreted in the urine within 24-48 h. Protease activity was also measured in buccal cells to evaluate usefulness as a biomarker. Single-dose BBI concentrate administered up to 800 CIU was well tolerated and appeared to be nontoxic.

Sun *et al.* (2010) isolated and purified two trypsin inhibitors from *Phaseolus vulgaris* cv “White Cloud Bean”. They reported the inhibition of [Methyl-3H] thymidine incorporation by leukemia L1210 cells with an $IC_{50}$ value of 28.8 μM and 21.5 μM.

Wang and Rao (2010) purified protease inhibitor from the large lima bean (*Phaseolus limensis*) legumes. They reported its antiproliferative activity toward tumor cells including human liver hepatoma cells Bel-7402 and neuroblastoma cells SHSY5Y.

Wati *et al.* (2010) isolated trypsin inhibitor from navy beans (*Phaseoulus vulgaris*), red kidney beans (*Phaseoulus vulgaris* L.), and adzuki beans (*Vigna angularis*) provided by the Royal Project Foundation in Thailand by heat and ammonium sulfate (AS) precipitation. Incubation at 70°C for 10 min produced the highest trypsin inhibitor recovery. The trypsin inhibitors had a molecular weight of 132 kDa for navy beans, 118 kDa for red kidney beans, and 13 kDa for adzuki beans under nonreducing conditions. The obtained precipitate IV fraction from each legume effectively prevented the degradation of the tilapia muscle with concentration dependent. The result indicated that the precipitate IV from these legumes have potential for use as a protease inhibitor in fish eryrelated products.

Hong *et al.* (2013) isolated and purified a novel trypsin inhibitor with considerable thermal and pH stability designated *Glytine*, from seeds of the Chinese black soybean *Glycine max* (L.) *Merr*. They reported that besides trypsin-chymotrypsin inhibition activity, *Glytine* demonstrated other biological activities like antiproliferative activity against tumor cells including human liver hepatoma cells Bel-7402 and neuroblastoma cells SHSY5Y.

Chan *et al.* (2014) purified a 16-kDa trypsin inhibitor from small pinto bean. They reported trypsin inhibitor slightly inhibited the viability of breast cancer MCF7 and hepatoma HepG2 cells at 125 mM.

Bezerra *et al.* (2016) purified a trypsin inhibitor from *Inga vera* seeds (IVTI), its biochemical and biological properties has also been described. Further assays revealed that IVTI is a chemopreventive agent against human epithelial colorectal adenocarcinoma cells (CACO-2), reducing cell viability by 70% at 200 g $mL^{-1}$.

Lima *et al.* (2016) reported inhibitory potential of the major seed protein fractions from eight selected legume species towards colon carcinoma cells. Albumin and globulin fractions were screened using a fluorometric assay and gelatin zymography. Effect of protein fractions on HT29 cell proliferation and cell migration was tested by them. Seed proteins include potent inhibitors, particularly low molecular mass proteins. Their effectiveness differs greatly among species, with a positive correlation detected between their inhibitory activity

and the reduction in cell migration. This may be important for selecting leguminous species with potential use in anti-cancer diets.

Li *et al.* (2017) studied the anticarcinogenic effect of purified 17.5-kDa trypsin inhibitor from *Phaseolus vulgaris* cv. “gold bean”. It inhibited [methyl-3H] thymidine incorporation by leukemia L1210 cells and lymphoma MBL2 cells with an $IC_{50}$ value of 2.3 µM and 2.5 µM, respectively.

Shamsi *et al.* (2017) obtained TIs from *Vigna unguiculata* “*lobia*” and *Allium sativum* “*garlic*”. By using MTT assay they was determined Antineoplastic potential on adenocarcinoma human alveolar basal epithelial cell line (A549) and normal Human Embryonic Kidney (HEK). They analyzed the result by spectrophotometer. They found that TIs (trypsin inhibitors) showed the higher cytotoxicity on A549 cells as compared to normal HEK cell line. TIs exhibited fair increase in antioxidant enzyme activity in A549 cells as compared to control. The results show that TIs possess ability to prevent cancer and diseases caused due to oxidative stress. Therefore, TIs can be used as supplements along with the conventional drugs for increased efficacy in the treatment of diseases such as cardiovascular disease, atherosclerosis, and cancer.

# *Chapter-3*

# MATERIALS AND METHODS

The present investigation on **"Studies on biological activity of Bowman-Birk trypsin inhibitor from a local bean cultivar"** was carried out in the Department of Biotechnology, Dr YS Parmar University of Horticulture and Forestry, Nauni, Solan (H.P.) during the year of 2016-2017. The details of the methods used have been described under the following headings:

## 3.1 MATERIALS

### 3.1.1 Seeds of local bean *(phaseolus vulgaris)* cultivar

Seeds of (*Phaseolus vulgaris* L.) cultivar Baspa **(Plate 1)** were procured from Sangla valley of Kinnaur, Himachal Pradesh, India.

### 3.1.2 Chemicals

The chemicals: Bovine pancreas trypsin, Sephadex G-100 and BApNA (α-Benzoyl–DL–arginine–p–nitroanilide) were purchased from Sigma Aldrich (USA). Molecular weight markers (SDS-markers 2 kDa – 40 kDa) were purchased from Merck (Germany) and rest of the chemicals used were from SRL Pvt. Ltd. (India).

### 3.1.3 Instruments

The following instruments were used during present studies:

1) Centrifuge (REMI)
2) Microfuge (REMI)
3) UV-1601(UV/VIS Spectrophotometer)
4) Hoefer Mini VE vertical electrophoresis system (Amersham Biosciences)
5) Columns (Pharmacia Biotech Pvt. Ltd.)
6) Hot water bath (Pharmacia)
7) Deep freeze -80$^{o}$C
8) Orbital incubator shaker (RIVOTEK)
9) Vortex mixer (REMI)
10) ELISA READER (MULTISCAN)

### 3.1.4 Fungal strain

Pure culture of five plant pathogenic fungal strains (viz., *Fusarium oxysporum, colletotrichum gloeosporiodium, Alternaria solani* and *Cercospora punicae*) were procured from the Department of Plant Pathology, Dr YS Parmar University of Horticulture and Forestry, Nauni, Solan, Himachal Pradesh.

### 3.1.5 Bacterial strain

Pure culture of three plant pathogenic bacterial strains (viz., *Ralstonia solanacearum, Agrobacterium tumefaciens and Xanthomonas campestris*) and two pathogenic bacterial strains (*Bacillus subtilis and Staphylococcus aureus*) were procured from the Department of Plant Pathology and Microbiology laboratory of the Department of Basic Sciences, Dr YS Parmar University of Horticulture and Forestry, Nauni, Solan, Himachal Pradesh.

### 3.1.6 Cancerous cell line

The Hep-2C cell line **i.e.** human cervix carcinoma cell line (Hela derivative) were procured from the Department of Biotechnology, HPU, Summer Hill, Shimla.

## 3.2 METHODS

### 3.2.1 Effect of extraction media on trypsin inhibitor activity

Seeds of local bean (*Phaseolus vulgaris* L.) cultivar Baspa were crushed and defatted with acetone (1:10 w/v) 3 times and air dried. Inhibitor protein was extracted in 10 ml (1:10 w/v) of different extraction media viz., distilled water, 0.1 M sodium phosphate buffer (pH 7.5), 0.1N NaCl and 0.1 N NaOH on magnetic stirrer for 3 hours at $4^{o}$C. The suspensions were centrifuged at 10,000 rpm for 20 minutes at $4^{o}$C. The supernatants were used for determination of trypsin inhibitor activity against bovine pancreas trypsin. Total soluble protein content was also estimated in the extracts.

### 3.2.2 Effect of extraction time on trypsin inhibitor activity

Seeds of local bean (*Phaseolus vulgaris* L.) cultivar Baspa were crushed, defatted and extracted in 10 ml of distilled water (pH 7.0) for 1, 2, 3 and 4 hours on magnetic stirrer at $4^{o}$C. The suspensions were centrifuged for 20 minutes at 10,000 rpm and used for the estimation of trypsin inhibitor activity and total soluble protein content.

**Plate 1. Seeds of local bean (*Phaseolus vulgaris* L. ) cultivar Baspa collected from Sangla valley of Kinnaur, Himachal Pradesh**

### 3.2.3 Trypsin inhibitor activity

Trypsin inhibitor activity was assayed in defatted seed flour of *Phaseolus vulgaris* L. cultivar by trypsin inhibition assay as described by Raj Deepika and Nath (2010).

#### 3.2.3.1 Reagents

**i) 0.1 M Sodium Phosphate buffer (pH 7.5)**

100 ml of phosphate buffer was made by mixing 16 ml of 0.1 M $NaH_2PO_4$ and 84 ml of 0.1 M $Na_2HPO_4$.

**ii) 0.1 M $NaH_2PO_4$**

1.19 g of $NaH_2PO_4$ was dissolved in minimum quantity of distilled water and the final volume was made to 100 ml with distilled water.

**iii) 0.1 M $Na_2HPO_4$**

1.42 g of $Na_2HPO_4$ was dissolved in minimum quantity of distilled water and the final volume was made to 100 ml with distilled water.

**iv) 3 M NaCl**

17.55 g of NaCl was dissolved in minimum quantity of distilled water and the final volume was made to 100 ml with distilled water.

**v) 0.1 M $CaCl_2$**

1.42 g of $CaCl_2$ was dissolved in minimum quantity of distilled water and the final volume was made to 100 ml with distilled water.

**vi) Buffer-I**

100 ml Buffer-I was prepared just before use by mixing: 50 ml of 0.1 M sodium phosphate buffer (pH 7.5), 20 ml of 0.1 M $CaCl_2$ and 30 ml of distilled water.

**vii) Buffer-II**

100 ml Buffer-II was prepared just before use by mixing: 0.1 M sodium phosphate buffer (pH 7.5), 5 ml of 0.1 M $CaCl_2$, 0.36 ml of 3 M NaCl and 44.64 ml of distilled water.

**viii) Trypsin Enzyme**

1.0 mg of bovine pancreas trypsin was dissolved in 10.0 ml of Buffer-II.

**ix) ~-Benzoyl-DL-arginine-p-nitroanilide (BApNA)**

4.0 mg of BApNA was dissolved in 0.1 ml of dimethyl sulphoxide (DMSO) by vortexing and the final volume was made to 1.0 ml by adding 0.9 ml of buffer-I.

**x) 30 per cent acetic acid**

Acetic acid solution was prepared by mixing: 30 ml of acetic acid with 70 ml of distilled water to make it 100 ml.

### 3.2.3.2 Trypsin inhibitor Assay

The assay mixture in a final volume of 2.0 ml contained 0.6ml of 0.1M phosphate buffer (pH 7.5), 1.0 ml of trypsin enzyme solution and 0.1 ml of inhibitor protein. The mixture was incubated at 37$^{o}$C for 5 minutes. The reaction was started by adding 0.3ml of substrate (BApNA). Reaction was stopped after 10 minutes by adding 1ml of 30 % acetic acid. Blank contained all the reagents except substrate which was replaced by equal volume of buffer. Control was prepared by excluding the inhibitor protein in the reaction mixture. Absorbance was recorded at 410 nm against the blank in a UV-VIS spectrophotometer.

### 3.2.4 Estimation of soluble protein

The total soluble protein content was estimated as described by Lowry *et al.* (1951).

### 3.2.4.1 Reagents

**i) 0.1 N NaOH**

400 mg of NaOH pellets were dissolved in 100 ml of distilled water.

**ii) 1% $CuSO_4$**

1 g of $CuSO_4$ was dissolved in 100 ml distilled water.

**iii) 2% $Na_2CO_3$**

2 g of $Na_2CO_3$ was dissolved in 100 ml of 0.1 N NaOH solution.

**iv) 2% $NaKC_4H_4O_6.4H_2O$**

2 g of $NaKC_4H_4O_6.4H_2O$ was dissolved in 10 ml of distilled water.

**v) 1 N Folin's Ciocalteau reagent**

25 ml of 1 N Folin's Ciocalteau reagent was mixed with 25 ml of distilled water. The reagent was prepared fresh just before use.

**vi) Reagent A**

The reagent A was prepared by mixing 50 ml of 2% $Na_2CO_3$ (dissolved in 0.1 N NaOH), 0.5 ml of 2% $NaKC_4H_4O_6.4H_2O$ and 0.5 ml of 1% $CuSO_4$. The reagent was prepared fresh just before use.

### 3.2.4.2 Procedure

5 ml of reagent A was added to 1 ml of suitably diluted protein solution and mixed immediately. 1 N Folin's reagent (0.5 ml) was added after 10 minutes and the test tube was shaken vigorously to ensure proper mixing. After 30 minutes, the intensity of color developed was measured at 660 nm against the reagent blank. The amount of protein was calculated from standard curve using bovine serum albumin (0-100 µg per ml).

### 3.2.5 Partial Purification of trypsin inhibitor protein

### 3.2.5.1 Extraction of inhibitor protein

The crude extract of *Phaseolus vulgaris* L. cultivar Baspa, was prepared in distilled water from defatted seed flour (1:20 w/v) of local bean (*Phaseolus vulgaris* L. ) cultivar Baspa.

### 3.2.5.2 Ammonium sulfate precipitation

The crude extract of *Phaseolus vulgaris* L. cultivar Baspa (5:100 w/v) was subjected to ammonium sulfate precipitation. The ammonium sulfate cuts of 0 to 20% and 20 to 80% were prepared by adding solid ammonium sulfate with constant stirring using magnetic stirrer at 4$^o$C. The protein was allowed to precipitate overnight at 4$^o$C. The precipitated protein was collected by centrifugation at 10,000 rpm for 20 minutes at 4$^o$C and the pellet was dissolved in minimum volume of distilled water.

### 3.2.5.3 Dialysis

The dialysis was carried out in dialysis bags. The dialysis bag was rinsed in distilled water and then boiled in excess of distilled water containing 50 µM EDTA to remove the impurities and eliminate release of ultraviolet light absorbing material during dialysis. The dialysis bag was washed thrice with distilled water. One end of the dialysis bag was tied with thread and the ammonium sulphate precipitates dissolved in distilled water were poured into the bag. The other end of dialysis bag was also tied and the precipitates were dialyzed overnight against distilled water.

### 3.2.5.4 Gel filtration Chromatography on Sephadex G-100 column

To obtain higher degree of purification inhibitor protein after ammonium sulphate precipitation was loaded on Sephadex G-100 column.

Sephadex G-100 (10 g) was suspended in 500 ml of distilled water for 48 hours. It was packed into the glass column having dimensions of (30x2.5) avoiding entrapment of any air bubble in the gel bed. It was then eluted with 3 bed volumes of distilled water. A flow rate of 12 ml per hour was maintained.

### 3.2.5.5 Void volume determination

Blue dextran solution (3 mg/ml) was loaded on the column and fractions of 3.0 ml were collected. The absorbance was recorded at 660 nm. This was done to check the uniformity of packed gel column and for calculating the void volume.

### 3.2.5.6 Loading of sample and collection of fractions

The precipitated protein (20-80 per cent ammonium sulfate fraction) 1 ml was loaded on the Sephadex G-100 column. Fractions of 3 ml were collected after discarding the void volume. It was eluted with 3 bed volumes of distilled water. The column was washed overnight with 5 bed volumes of distilled water before loading next lot of ammonium sulfate cut. The fractions were analyzed for protein content at 280 nm and for trypsin inhibitor activity. The most active fractions were pooled and stored at $4^{o}$C. The purity of different fractions were judged by SDS-PAGE.

### 3.2.5.7 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

The purity of partially purified trypsin inhibitor protein obtained after purification was checked by Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) on vertical slab gel using 12% acrylamide gels and the method adopted was as described by Laemmli (1970).

#### 3.2.5.7.1 Reagents

**i) Acrylamide-bisacrylamide stock solution**

| | |
|---|---|
| Acrylamide | 30.0 g |
| Bisacrylamide | 0.8 g |

Dissolved in water and the final volume was made to 100 ml with distilled water. Filtered the solution through Whatman No. 1 filter paper and stored in brown bottle at 0-4$^{o}$C.

**ii) Stacking gel buffer stock (Tris-HCl, pH-6.8)**

| | |
|---|---|
| Tris | 6.0 g |
| 1 M HCl | 48.0 ml |

Adjusted its pH to 6.8 and the final volume was made to 100 ml with distilled water. Filtered it through Whatman No. 1 filter paper and stored at 0-4$^{o}$C.

**iii) Resolving gel buffer stock (Tris-HCl, pH 8.8)**

| | |
|---|---|
| Tris | 36.3 g |
| 1 M HCl | 48.0 ml |

Adjusted its pH to 8.8 and the final volume was made to 100 ml. It was filtered through Whatman No. 1 filter paper and stored at 0-4$^{o}$C.

**iv) 1.5 % (w/v) Ammonium persulfate (APS)**

This solution was prepared by dissolving 0.15 g of APS in 10 ml of distilled water. This reagent was prepared fresh just before use.

**v) Staining solution**

| | |
|---|---|
| Coomassie brilliant blue R-250 | 1.25 g |
| Methanol | 200 ml |
| Glacial acetic acid | 35 ml |

After mixing the above components, the final volume was made to 500 ml with distilled water. It was filtered to remove undissolved materials and stored at room temperature.

**vi) Destaining solution**

| | | |
|---|---|---|
| Glacial acetic acid | - | 75 ml |
| Methanol | - | 50 ml |

The above components were mixed and the final volume was made to one litre with 875 ml distilled water.

**vii) Reservoir buffer (Tris-glycine, pH 8.3)**

| | |
|---|---|
| Tris | 3.0 g |
| Glycine | 14.4 g |
| SDS | 1.0 g |

Dissolved in distilled water (800 ml) and adjusted its pH to 8.3. The final volume was made to 1 litre with distilled water.

**viii) 10 % (w/v) Sodium dodecyl sulphate**

SDS (10 per cent, w/v): 1 g of SDS was dissolved in 10 ml of distilled water. This reagent was stored at room temperature

**ix) Sample buffer - 2 X**

| | |
|---|---|
| 1 M Tris-HCl, pH 6.8 | 12.5 ml |
| SDS | 4.0 g |
| β-Mercaptoethanol | 10.0 ml |
| Glycerol | 20.0 ml |
| 1 per cent Bromophenol blue | 4.0 ml |

Distilled water was added to make the final volume to 100 ml.

**3.2.5.7.2 Sample preparation**

The partially purified trypsin inhibitor was mixed with equal volume of sample buffer. The mixture was then boiled for 3 minutes in a boiling water bath and cooled at room temperature.

**3.2.5.7.3 Preparation of slab gel and electrophoresis of sample**

Glass plates were washed with distilled water and oven dried. The plates were placed on gel casting assembly. The plates were clamped properly avoiding any leakage and air bubble entrapment. Various components of the resolving gel were mixed as specified in the Table 1. and were poured into glass plate assembly after addition of TEMED. About 20-30µl

of partially purified protein sample was loaded in sample wells. The electrophoresis was carried out by maintaining current of 2 mA per well was maintained until bromophenol blue reached the other end of the slab gel. After the electrophoresis was complete, the power supply was disconnected and the gel was removed carefully and was placed in staining solution for 3-4 hours. The gel was destained by changing the destaining solution frequently.

**Table 1. Composition of stacking and resolving gel for SDS-PAGE**

| Stock Solution | Stacking gel (2.5 %) | Resolving gel (12.5 %) |
|---|---|---|
| | ml of the solution | |
| Acrylamide-bis acrylamide stock solution | 2.50 | 12.50 |
| Stacking gel buffer (Tris HCl pH 6.8) | 5.00 | - |
| Resolving gel buffer (Tris HCl, pH 8.8) | - | 3.75 |
| 10 per cent SDS | 0.20 | 0.30 |
| 1.5 per cent APS | 1.00 | 1.50 |
| Water | 11.30 | 11.95 |
| TEMED | 0.015 | 0.015 |
| **Total volume** | **20.00** | **30.00** |

### 3.2.6 Antimicrobial activity of partially purified inhibitor protein

#### 3.2.6.1 Effect of partially purified inhibitor protein on fungal growth

PDA (potato dextrose agar) (3.9:100 w/v) medium was prepared in distilled water in different flasks. Each flask contained 25ml of medium. Then the medium was autoclaved. The medium was cooled to 35-40°C and different concentrations of filter sterilized partially purified inhibitor protein (µg/ml) was added to this. The medium was mixed well under laminar airflow chamber. In control equal volume of autoclaved distilled water was added to the medium. The medium was allowed to solidify.

A bit of fungus having diameter 8 mm was cultured on PDA medium in Petri plates containing different concentrations of partially purified inhibitor protein. In control inhibitor protein was not added in the PDA medium. The Petri plates were incubated at 28$^{o}$C for further growth. The diameter of growing fungus was measured after 7 days of culturing and percent inhibition as compared to control was calculated.

#### 3.2.6.2 Effect of partially purified inhibitor protein on bacterial growth

##### 3.2.6.2.1 Preparation of inoculum

2.50 g of nutrient broth (NB) medium was dissolved in 100 ml of distilled water and heated to dissolve completely. The medium was poured into test tubes and autoclaved.

Bacterial suspension was grown in the NB medium by inoculating the autoclaved medium with the bacterial strain using inoculating loop under laminar air flow chamber. A bacterial cell suspension (100µl) of 48 hours old culture grown on nutrient broth was used as inoculum in further experiments.

#### 3.2.6.2.2 Growth inhibition of bacterial strain

Nutrient Agar (NA) medium (3.5:100 w/v) was used for the plating bacterial strains. After plating, different concentrations (µg/ml) of partially purified inhibitor protein was loaded in one well of the 2 wells made on each petri plates and to the remaining well, distilled water was loaded . The Petri plates were then incubated at $37^{o}$C for further growth. After incubation for 24 hours, plates were observed to see the effect of partially purified inhibitor on bacterial growth.

### 3.2.7 Anticancerous activity of partially purified inhibitor protein

#### 3.2.7.1 Cell proliferation assay (MTT assay)

To determine cell viability the colorimetric MTT metabolic activity assay was used. Hep-2C cell lines ($1.0 \times 10^4$/ml) were cultured in a 96-well plate at 37 °C, and exposed to varying concentrations (µg/ml) of partially purified inhibitor protein for 24h. Cells treated with distilled water only served as a control group. After removing the supernatant of each well and washing twice by PBS (Phosphate buffer saline), 20 µl of MTT solution (5 mg $ml^{-1}$ in PBS) were then introduced. After incubation for another 3-4 h, the resultant formazan crystals were dissolved in dimethyl sulfoxide (100 µl) and the absorbance intensity measured by a ELISA reader (MULTISCAN EX; Thermo Fisher Scientific, China) at 570 nm. All experiments were performed in replicates, and the relative cell viability (%) was expressed as a percentage relative to the untreated control cells.

#### 3.2.7.2 Per cent cell viability

To calculate cell viability, absorbance of the blank was subtracted from absorbance of all samples. Absorbance from test samples then were divided by those of the control and multiplied by 100 to give percentage cell viability or proliferation. Absorbance values greater than the control indicated cell proliferation, while lower values suggested cell death or inhibition of proliferation.

$$\%\,viability = \frac{abs(sample) - abs(blank)}{abs(cantrol) - abs(blank)} \times 100$$

### 3.2.8 STATISTICAL ANALYSIS

All the laboratory experiments were carried out in three replications with duplicate for each replication. The CRD design was applied to all laboratory experiments (Gomez and Gomez, 1984).

# *Chapter-4*

# RESULTS AND DISCUSSION

The following observations were recorded during the course of investigation **"Studies on biological activity of Bowman-Birk trypsin inhibitor from a local bean cultivar"** and discussed in the light of available literature:

## 4.1 EFFECT OF DIFFERENT EXTRACTION MEDIA ON TRYPSIN INHIBITOR ACTIVITY

Four different extraction media were used to extract trypsin inhibitor from seeds of local bean (*Phaseolus vulgaris* L.) cultivar Baspa. Maximum inhibitor activity was obtained in distilled water (2893.33±2.09) and minimum in 0.1 N NaCl (1546.66±1.42). Specific inhibitor activity was also maximum in distilled water (20.63±0.23) and minimum in 0.1 N NaCl (13.17±0.03). The results are shown in Table 2.

**Table 2. Effect of different extraction media on trypsin inhibitor activity**

| Extraction media | Total inhibitor activity (TUI/g seed weight) | Total soluble protein (mg/g seed weight) | Specific inhibitor activity (TUI/mg protein) |
|---|---|---|---|
| **0.1 M Phosphate buffer (pH 7.5)** | 2596.66±0.90$^{b}$ | 130.22±0.40$^{b}$ | 19.93±0.0$^{b}$ |
| **Distilled water** | 2893.33±2.09$^{a}$ | 138.44±0.20$^{a}$ | 20.63±0.23$^{a}$ |
| **0.1 N NaCl** | 1546.66±1.42$^{d}$ | 117.33±0.20$^{d}$ | 13.17±0.03$^{d}$ |
| **0.1 N NaOH** | 2023.33±1.30$^{c}$ | 122.44±0.23$^{c}$ | 16.55±0.02$^{c}$ |
| **$CD_{0.05}$** | 4.88 | 1.02 | 0.39 |

Data represents mean values ± standard error of three values.
Values in the same column followed by a similar superscript letters are not significantly different at $p \leq 0.05$.

Same extraction media was used for the extraction of inhibitor protein from seed flour of *Glycine soya, Phaseolus vulgaris, Vigna radiata* L.Wilczek and *Vicia faba* (Zhang *et al.*, 2009; Raj Deepika and Nath., 2010; Klomklao *et al.*, 2011 and Sharma 2013). Wang *et al*. (2006) used 0.2 M sodium acetate buffer (pH-5.4) for the extraction of protease inhibitor from the seeds of *Phaseolus mungo*. Nath *et al*. (2014) extracted trypsin inhibitor from the seed flour of *D. biflorus* cultivar HPK4 in 0.1 M sodium phosphate buffer (pH-7.5) and Chilosi *et al*. (2000) used 0.15 M NaCl for the extraction of trypsin inhibitor from wheat kernel. However, 100

mM potassium phosphate (pH-7.6) for the extraction of trypsin inhibitor from the seeds of *Inga vera* was used by Bezerra *et al.* (2016).

## 4.2 EFFECT OF EXTRACTION TIME ON TRYPSIN INHIBITOR ACTIVITY

For the extraction of inhibitor protein from seeds of local bean (*Phaseolus vulgaris* L.) cultivar Baspa, distilled water was used as extraction media and extraction time was varied from 1-4 hours. Maximum inhibitor activity and specific activity was obtained in 3 hours of extraction time (2953.33±0.02 and 21.13±0.01) and minimum in 1 hour (1453.33±0.40 and 13.94±0.00). The results are presented in Table 3.

**Table 3. Effect of extraction time on trypsin inhibitor activity**

| Extraction time (Hour) | Total inhibitor activity (TUI/g seed weight) | Total soluble protein (mg/g seed weight) | Specific inhibitor activity (TUI/mg protein) |
|---|---|---|---|
| **1 hour** | 1453.33±0.40$^{d}$ | 104.22±0.07$^{d}$ | 13.94±0.00$^{d}$ |
| **2 hours** | 2686.66±0.50$^{b}$ | 129.99±0.19$^{b}$ | 20.66±0.01$^{b}$ |
| **3 hours** | 2953.33±0.02$^{a}$ | 139.77±0.09$^{a}$ | 21.13±0.01$^{a}$ |
| **4 hours** | 2046.66±0.28$^{c}$ | 114.44±0.04$^{c}$ | 17.88±0.01$^{c}$ |
| **$CD_{0.05}$** | 0.92 | 0.38 | 0.04 |

Data represents mean values ± standard error of three values.
Values in the same column followed by a similar superscript letters are not significantly different at $p \leq 0.05$.

Inhibitor protein from seed flour of *Vigna unguiculata* and *Vicia faba* was also extracted for 3 hours by (Gupta *et al*., 2000 and Sharma 2013). Klomklao *et al*. (2011), Satheesh and Murugan, (2011) extracted trypsin inhibitor protein from seed flour of *Vigna radiata* L. Wilczek and leaves of *Coccinia grandis* for 2 hours respectively. Extraction time of 2 hours was used by Terras *et al*. (1993) for the extraction of trypsin inhibitor from seedflour of *B.napus, B. rapa* and *Hordeum vulgare.* Extraction time of 4 hours was reported by Raj Deepika *et al*. (2008) for the extraction of inhibitor protein from the seeds of *Phaseolus vulgaris* L. Pesoti *et al*. (2015) extracted inhibitor protein from the seeds of *Chinopodium quinoa* for 5 hours. In contrast, seed flour of *Cajanus cajan* were stirred overnight for the extraction of inhibitor protein (Swathi *et al*., 2014).

## 4.3 PARTIAL PURIFICATION OF TRYPSIN INHIBITOR FROM SEEDS OF *Phaseolus vulgaris* L. CULTIVAR BASPA ON SEPHADEX G-100 COLUMN

The void volume of Sephadex G-100 column was calculated by loading blue dextran solution (3 mg per ml) on to the column. Fractions of 3 ml were collected and optical density

was measured at 660 nm. The void volume was found to be 33 ml. The elution profile of blue dextran is shown in Fig. 1. To achieve higher degree of purification the ammonium sulphate fraction (20-80 %) after dialysis was loaded on to Sephadex G-100 column. After discarding the void volume, fractions of 3 ml were collected and analyzed for protein content and trypsin inhibitor activity. The elution profile of inhibitor protein obtained on Sephadex G-100 column is shown in Fig. 2. The most active fractions were pooled and used for further studies. The data of total trypsin units inhibited (TUI), protein content, specific activity, fold purification and yield at different stages of purification are presented in Table 4. The trypsin inhibitor was partially purified to 5.5 fold with 76.5 % recovery. The specific activity increased from 19.04 TUI/mg protein in crude extract to 105.98 TUI/mg in partially purified inhibitor protein from Sephadex G-100 column.

**Table 4. Partial purification of trypsin inhibitor from *Phaseolus vulgaris* L. cultivar Baspa on Sephadex G-100 column**

| Steps | Total Inhibitor Activity (TUI) | Total soluble protein (mg) | Specific activity (TUI/mg protein) | Fold purification | Percent yield |
|---|---|---|---|---|---|
| **Crude extract** | 14166 | 744.2 | 19.04 | 1 | 100 |
| **Ammonium sulfate precipitation** | 12833 | 318.8 | 40.25 | 2.1 | 90.6 |
| **Gel filtration chromatography** | 10833 | 102.2 | 105.98 | 5.5 | 76.5 |

Sharma *et al*. (2012) purified trypsin inhibitor from seeds of *Albizia lebbeck* to 3.07 fold with 74.07 % recovery using ammonium sulphate precipitation and gel filtration chromatograpy. Trypsin inhibitor protein was purified from seeds of *Inga vera* to 1.36 fold with 7.14 per cent recovery using DEAE Sepharose and trypsin Sepharose (Bezerra *et al*., 2016). Klomklao *et al*. (2011) purified trypsin inhibitor to 13.51 fold with 30.25 % recovery from seeds of *Vigna radiata* L. Wilczek by ammonium sulfate precipitation (30-65%) and gel filtration chromatography on Sephadex G-50. Protease inhibitor protein from leaves of *Cassia fistula* was purified to 9.2 fold with 15.4 % recovery by ammonium sulphate precipitation and Sephadex G-100 (Arulpandi and Sangeetha, 2012). Thakur (2004) purified trypsin inhibitor to 10.05 fold with 48.25 % recovery by ammonium sulphate precipitation, ion exchange chromatography and gel filteration chromatography.

The purity of the final preparation of partially purified trypsin inhibitor from Gel filtration chromatography was checked by Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) on vertical slab gel using 12% acrylamide gel (Laemmli, 1970).

Seven bands were obtained when crude extract protein was subjected to SDS-PAGE. Six bands were obtained when ammonium sulfate precipitated protein was subjected to SDS-PAGE. Two bands were obtained when partially purified inhibitor protein was subjected to SDS-PAGE. The numbers of bands after partial purification were considerably less as compared to crude extract and ammonium sulfate protein. To calculate the molecular weight of the partially purified trypsin inhibitor, known protein molecular weight ladder having molecular weight 250000, 80000, 50000, 30000, 25000, 20000, 15000, 10000 daltons was used. The molecular weight of bands obtained on SDS-PAGE was approximately 12000 and 14000 daltons, respectively. Results of SDS-PAGE are shown in Plate 2. Pesoti *et al*. (2015) reported trypsin inhibitor from seeds of *Chenopodium quinoa* seeds to have molecular weight of approximately 12 kDa, which supported our finding that it is Bowman-Birk trypsin inhibitor protein . Kuhar *et al*. (2014) reported the molecular mass of *D. biflorus* PI as 16 kDa both by SDS-PAGE and gel filtration chromatography which indicated that the inhibitor consisted of a single polypeptide chain. Godbole *et al*. (1994) found two PIs, viz. trypsin-chymotrypsin inhibitor and trypsin inhibitor (Bowman-Birk type) with molecular weights of 15 and 10.5 kDa, respectively in pigeon pea. Raj Deepika and Nath (2010) reported purified trypsin inhibitor from bean to have a molecular weight of 14.13 kDa by SDS-PAGE. Prasad *et al*. (2010) purified proteinase inhibitor from red gram with molecular mass of 8,500 daltons and 16,500 daltons corresponding to monomeric and dimeric forms. Purified trypsin inhibitor from seeds of *Chenopodium quinoa* seeds had molecular weight of approximately 12 kDa (Pesoti *et al*., 2015).

## 4.4 BIOLOGICAL ACTIVITY OF PARTIALLY PURIFIED TRYPSIN INHIBITOR

### 4.4.1 Antimicrobial activity of partially purified trypin inhibitor

#### 4.4.1.1 Effect of partially purified inhibitor protein on growth of different fungal strains

Antifungal activity of partially purified trypsin inhibitor was tested against four different types of fungus viz., *Fusarium oxysporum, Colletotrichum gloeosporioides, Alternaria solani and Cercospora punicae.* The pathogenic fungi and disease symptoms caused by them are enlisted in Table 5 and Plate 3.

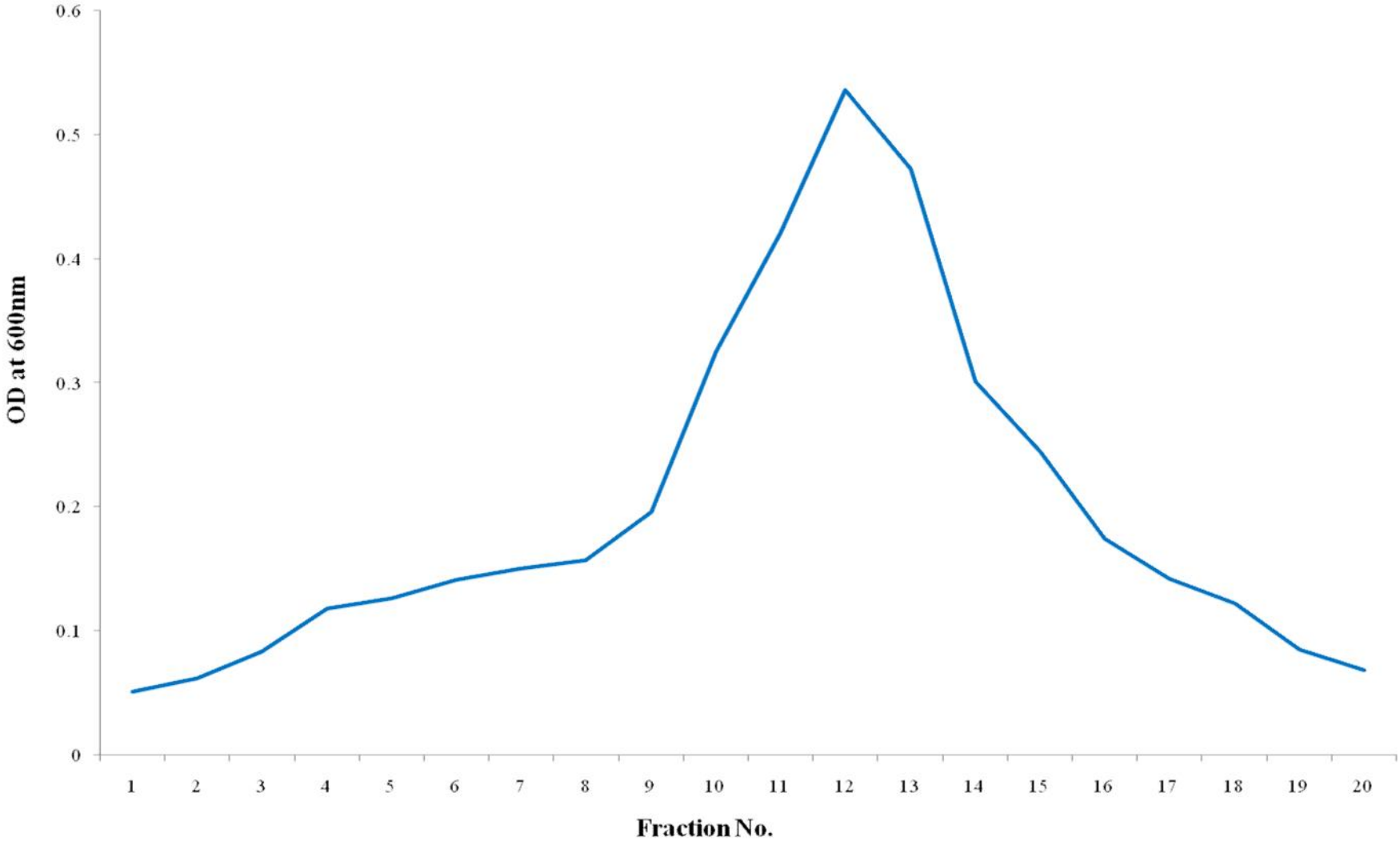


**Fig. 1 Elution profile of Blue dextran on Sephadex G-100**

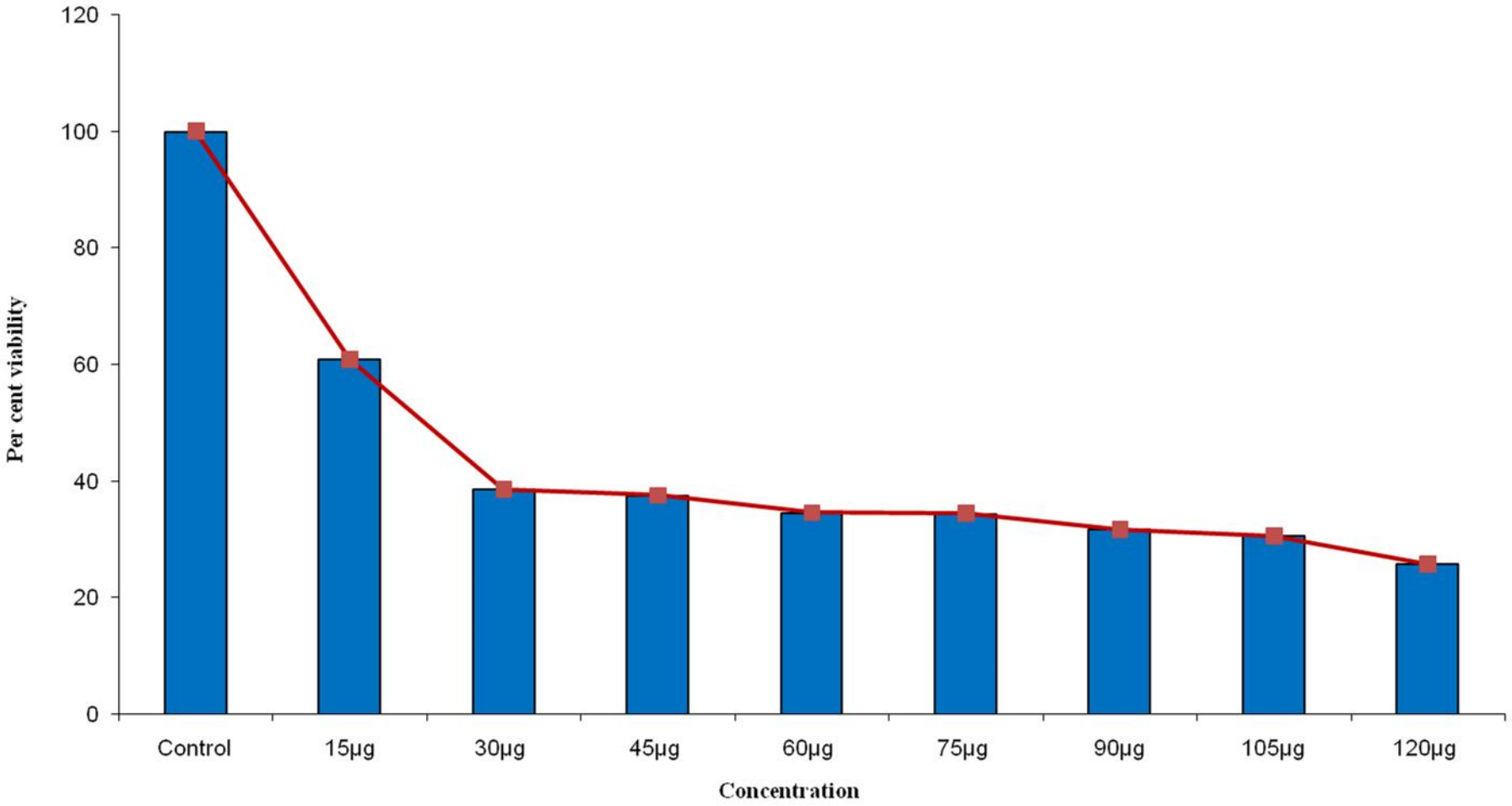


**Fig. 3 Per cent viability of Cancerous cells (Hep-2C cell line) at different concentration of inhibitor protein**

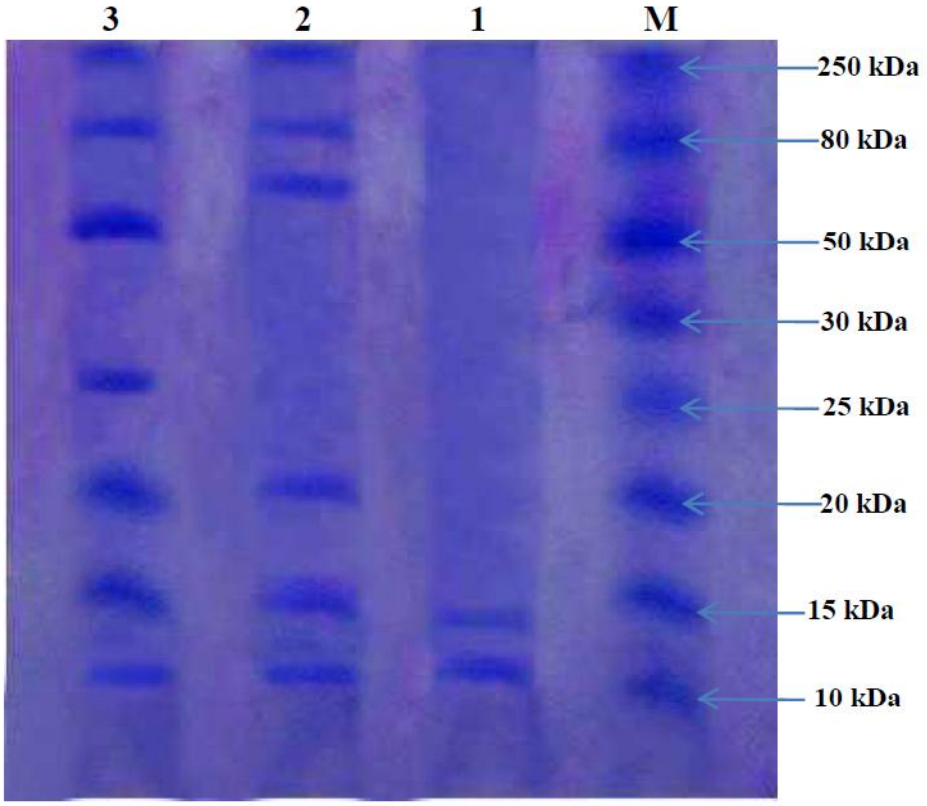


**Plate 2. SDS-PAGE of protein patterns at different steps of purification**

| | | |
|---|---|---|
| **M** | : | SDS-PAGE molecular weight marker |
| **Lane 1** | : | Partially purified inhibitor protein |
| **Lane 2** | : | Ammonium sulphate fraction of inhibitor protein |
| **Lane 3** | : | Crude inhibitor protein |


**Plate 3. Disease symptoms of pathogenic fungi on plants**

(a) Tomato plant affected by *Fusarium oxysporum* with brown stem and wilted leaves
(b) Mango anthracnose affected by *Colletotrichum gloeosporioides* with black spots
(c) Early blight of potato caused by *Alternaria solani,* Potato tuber develop dark, sunken lesions, under these lesions, the tissue is dry and brown
(d) Light brown spots on the leaves and fruits of pomegranate caused by *Cercospora punicae*

**Table 5. Disease symptoms of pathogenic fungi on plants**

| Plant pathogenic fungi | Disease caused | Disease symptoms |
|---|---|---|
| *Fusarium oxysporum* | Fusarium wilt of tomato | Yellowing on one side of the plant or leaf, followed by wilting, browning and defoliation |
| *Colletotrichum gloeosporioides* | Mango anthracnose | Infected fruit develop black spots, shrivel and drop off, cause considerable loss during storage and marketing |
| *Alternaria solani* | Early blight of potato | Potato tuber develop dark, sunken lesions, under these lesions, the tissue is dry, leathery and brown |
| *Cercospora punicae* | Cercospora leaf and fruit spot on pomegranate | Light brown spots appears on the leaves and fruits, Black and elliptic spots appear on the twigs |

All these fungus showed high growth in PDA (potato dextrose agar) medium not supplemented with inhibitor protein. As the concentration of inhibitor protein was increased in the medium the growth of fungal strain decreased in case of each fungal culture **(Plate 4, 5, 6 and 7)**. Maximum per cent (88.12%) inhibition of growth was observed in fungal strain *Cercospora punicae* in medium (PDA) supplemented with 300 µg of inhibitor protein. While at the same concentration of inhibitor protein in medium, 84.20%, 81.00% and 79.41% inhibition in growth was found in case of *Fusarium oxysporum*, *Colletotricum gloeosporioides* and *Alternaria solani* respectively. Minimum per cent inhibition in growth (9.52%) was observed in fungus *Alternaria alternata* followed by *Fusarium oxysporum* (15.21%), *Cercospora punicae* (17.14%) and *Colletotrichum gloeosporioides* (29.16%) when the strains were cultured on the medium supplemented with 60 µg of inhibitor protein. The results obtained are presented in Table 6.

Protease inhibitors are known to inhibit the growth of several phytopathogenic fungi. They affect the fungus by inhibiting extracellular and intracellular proteases that display important roles in nutrition and infection processes since the invasion of host tissue and fungal development depends on the degradation of membrane and cell wall proteins. Thus the protease inhibitors act directly on the protease produced by pathogenic fungi and reduce their pathogenicity. Literature has been found regarding antifungal activities of Protease inhibitor. Chilosi *et al.* (2000) reported a strong antifungal activity *in vitro* by inhibiting hyphal growth of different fungi, *Septoria tritici*, *Fusarium graminearum* and *Fusarium culmorum* in presence of inhibitor protein isolated from wheat kernels.

**Table 6. Effect of partially purified trypsin inhibitor on fungal growth**

| Inhibitor concentration (µg) | Per cent inhibition of fungal growth (%) | | | |
|---|---|---|---|---|
| | ***Fusarium oxysporum*** | ***Colletotrichum gloeosporioides*** | ***Alternaria Solani*** | ***Cercospora punicae*** |
| **60** | 15.21±0.21 | 29.16±0.12 | 9.52±0.20 | 17.14±0.18 |
| **120** | 27.42±0.13 | 36.03±0.09 | 20.17±0.15 | 34.03±0.12 |
| **180** | 43.25±0.12 | 52.28±0.12 | 41.52±0.14 | 53.20±0.31 |
| **240** | 60.00±0.20 | 63.14±0.16 | 52.64±0.09 | 67.75±0.16 |
| **300** | 84.20±0.17 | 81.00±0.42 | 79.41±0.19 | 88.12±0.18 |
| **$CD_{0.05}$** | 0.55 | 0.70 | 0.52 | 0.65 |

Data represents mean values ± standard error of three values.

Wang and Ng (2007) isolated an antifungal peptide from *P. vulgaris* cv. 'Spotted Bean', which exhibited antifungal activity against *Fusarium oxysporum* and *Mycosphaerella arachidicola*. Antifungal activity of protease inhibitor isolated from *Acacia plumose* was reported by Lopes *et al*. (2009) and they observed antifungal activity against *Aspergillus niger* and *Fusarium moniliforme* hyphae. Dias *et al*. (2012) reported morphological changes including cellular agglomeration and formation of pseudohyphae in *Candida tropicalis* when subjected to inhibitor protein isolated from *Capsicum chinere.* Antifungal activity of trypsin inhibitor protein isolated from *Abelmoschus moschatus* seeds (Dokka and Davuluri, 2014), was reported against *Candida albicans, Candida tropicalis, Asperigillus flavus, Saccharomyces cerevisiae, Candida glabrata* and *Asperigillus niger.* Barla *et al.* (2016) reported antifungal activity of crude inhibitor extract isolated from testa of *Citrullus lanatus* Linn. *against* the growth of fungus *Asperigillus niger* and *Candida albicans.* Dabhade *et al.* (2016) reported antifungal activity of nontoxic trypsin inhibitor protein isolated from *Albizia amara* Boiv. against *Alternaria alternata, Alternaria tenuissima* and *Candida albicans.*

### 4.4.1.2 Effect of partially purified trypsin inhibitor on bacterial growth

Effect of partially purified trypsin inhibitor protein isolated from *Phaseolus vulgaris* L. cultivar Baspa was tested against 3 different types of plant pathogenic bacterial strains viz., *Xanthomonas campestris, Agrobacterium tumefaciens* and *Ralstonia solanacearum* & 2 non-plant pathogenic bacterial strains viz., *Bacillus subtilis* and *Staphyloccocus aureus*. The disease symptoms caused by them are shown in Table 7.

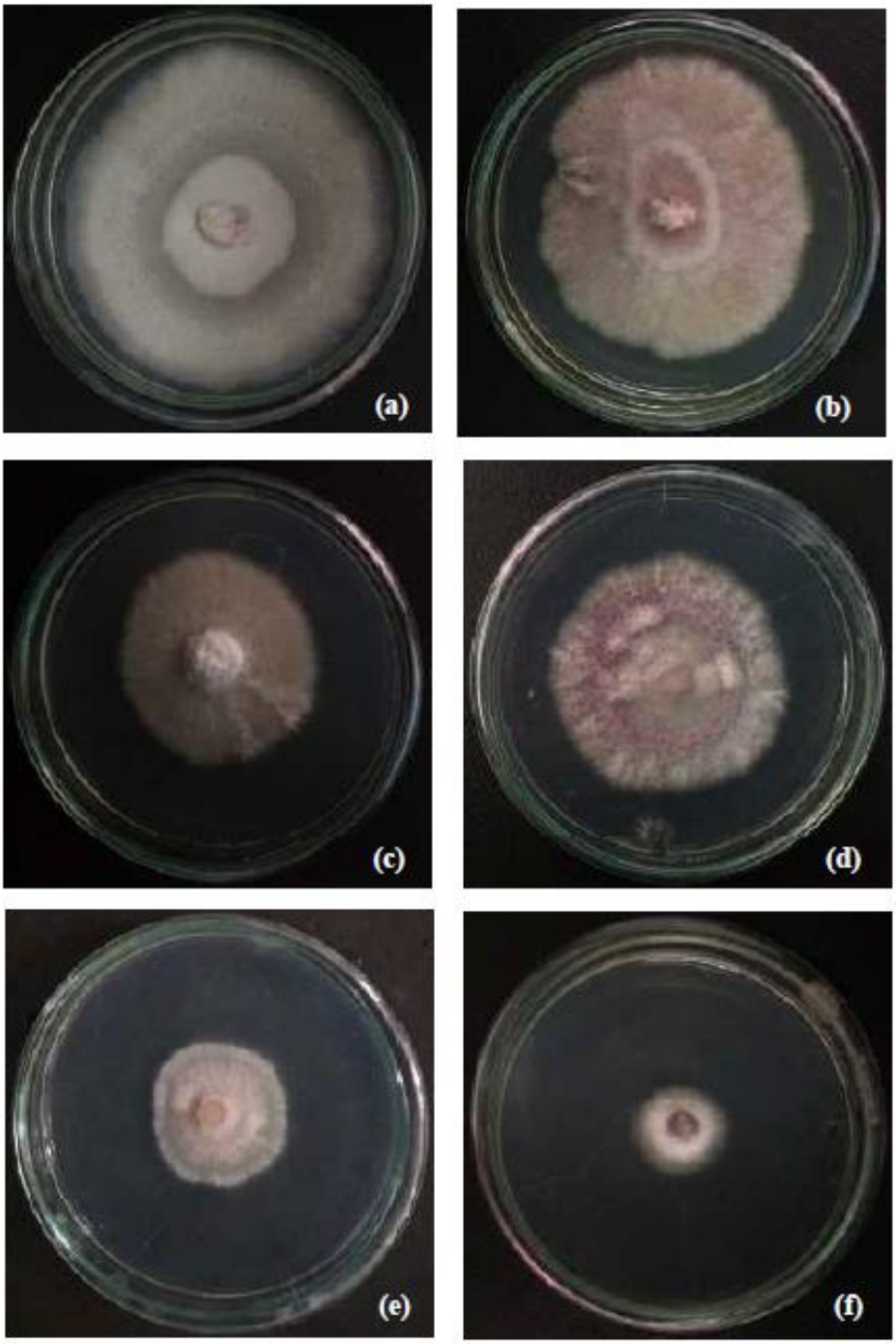


**Plate 4. Antifungal activity of partially purified inhibitor on *Fusarium oxysporum* growth at different concentrations of inhibitor protein in the growth medium**

(a) Control = 0 µg
(b) Inhibitor conc.= 60µg
(c) Inhibitor conc.= 120µg
(d) Inhibitor conc.= 180µg
(e) Inhibitor conc.= 240µg
(f) Inhibitor conc.= 300µg

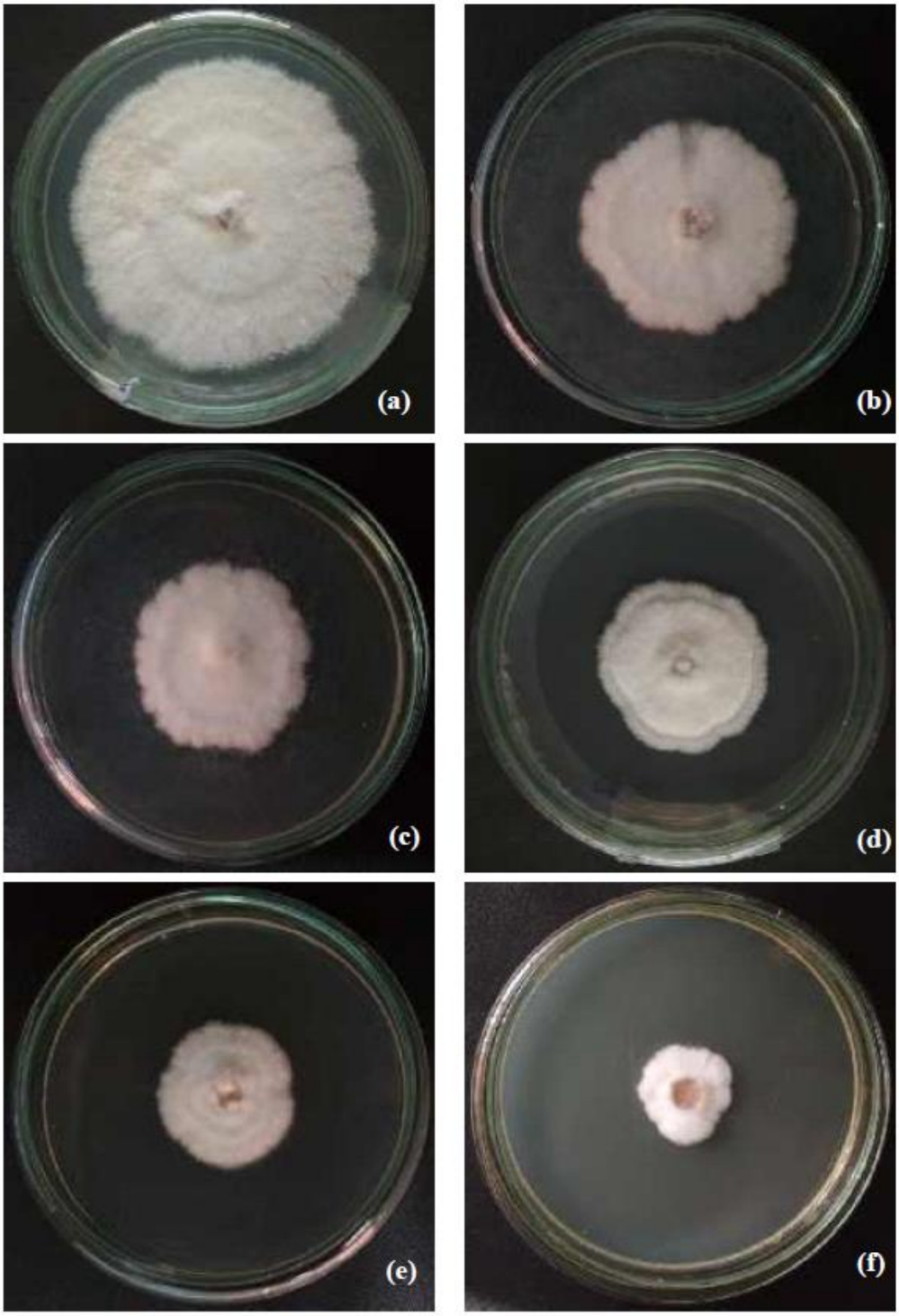


**Plate 5. Antifungal activity of partially purified inhibitor on *Colletotrichum gloeosporioides* growth at different concentrations of inhibitor protein in the growth medium**

(a) Control = 0 µg
(b) Inhibitor conc.= 60µg
(c) Inhibitor conc.= 120µg
(d) Inhibitor conc.= 180µg
(e) Inhibitor conc.= 240µg
(f) Inhibitor conc.= 300µg

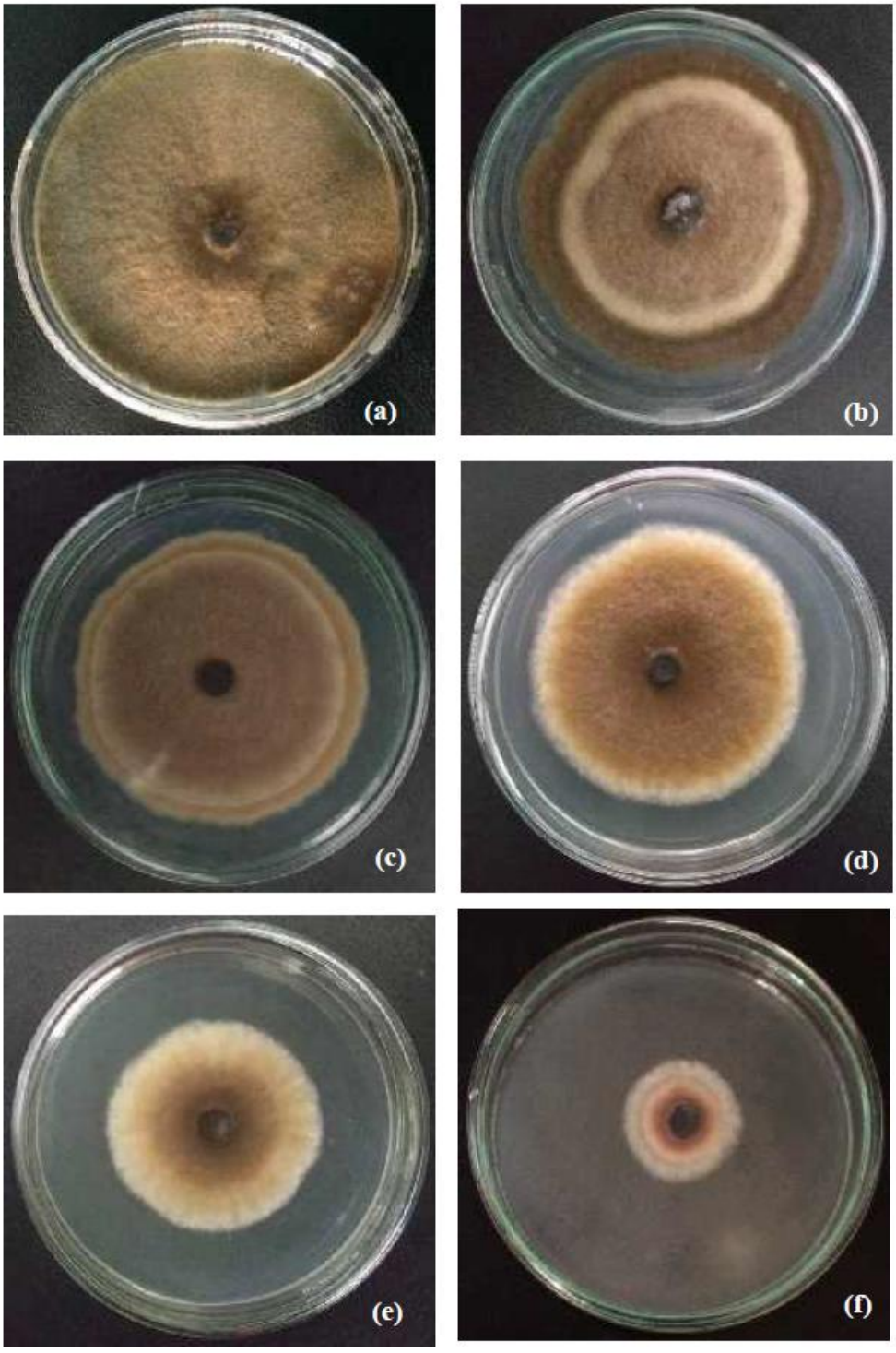


**Plate 6.** **Antifungal activity of partially purified inhibitor on *Alternaria alternata* growth at different concentrations of inhibitor protein in the growth medium**

(a) Control = 0 µg
(b) Inhibitor conc.= 60µg
(c) Inhibitor conc.= 120µg
(d) Inhibitor conc.= 180µg
(e) Inhibitor conc.= 240µg
(f) Inhibitor conc.= 300µg

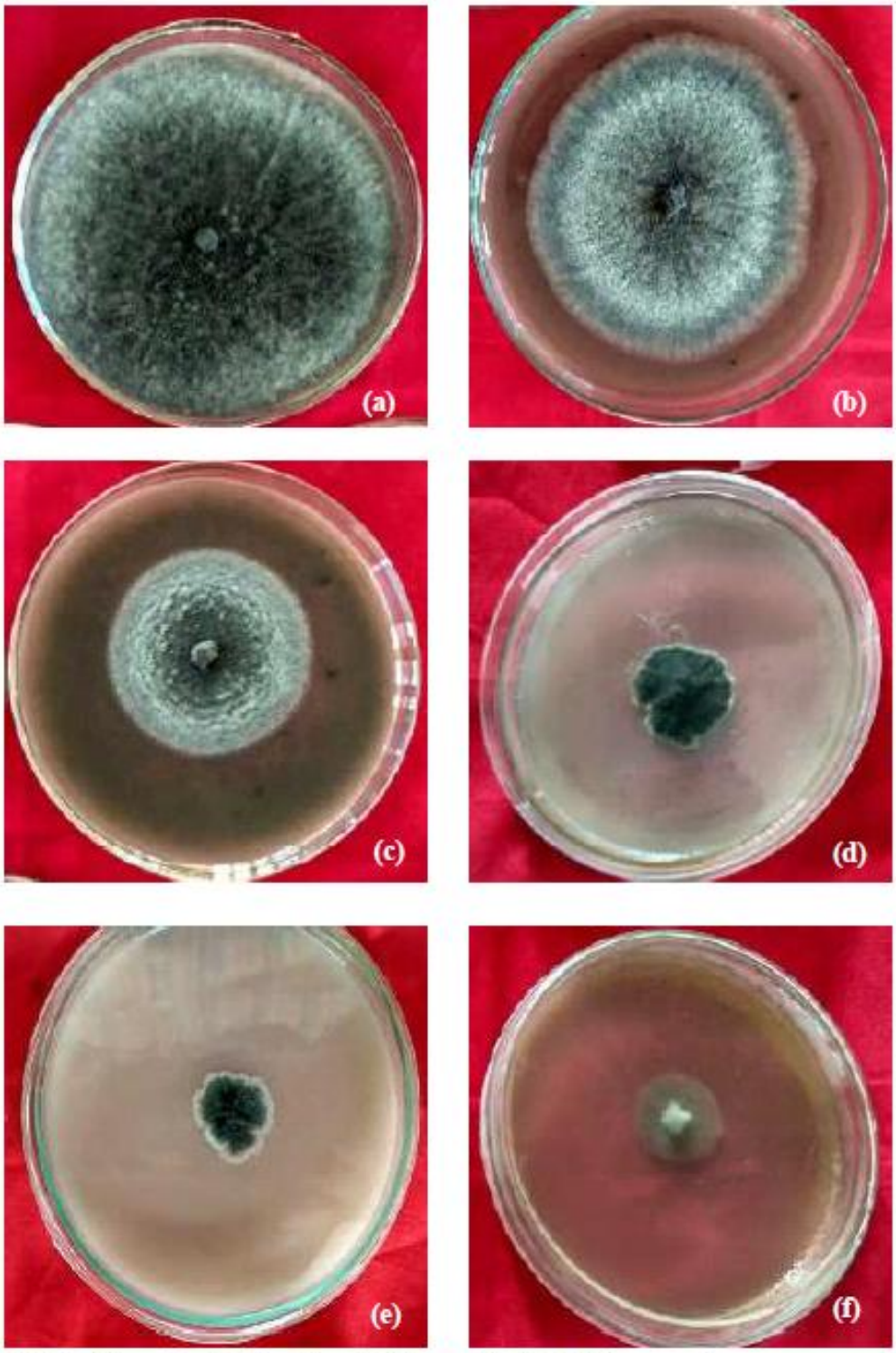


**Plate 7. Antifungal activity of partially purified inhibitor on *Cercospora punicae* growth at different concentrations of inhibitor protein in the growth medium**

(a) Control = 0 µg
(b) Inhibitor conc.= 60µg
(c) Inhibitor conc.= 120µg
(d) Inhibitor conc.= 180µg
(e) Inhibitor conc.= 240µg
(f) Inhibitor conc.= 300µg

**Table 7. Disease symptoms of pathogenic bacteria on plants and other organisms**

| Plant pathogenic bacteria | Disease caused | Disease symptoms |
|---|---|---|
| *Xanthomonas campestris* | Black rot of cabbage | V-shaped yellow lesions to margin of leaves, vascular blackening, stem rot and wilting |
| *Agrobacterium tumefaciens* | Crown gall disease of apple | Galls form on the roots or stems and have a rough convoluted surface |
| *Ralstonia solanacearum* | Bacterial wilt of tomato | wilting of tomato leaves without their yellowing and stunted growth |
| **Other bacteria** | **Symptom caused** | |
| *Staphyloccocus aureus* | Various skin infections and food poisoning | |
| *Bacillus subtilis* (non-pathogenic) | Food contamination results in food poisoning | |

The inhibitor protein was highly effective against bacterial strain *Bacillus subtilis* and less effective against *Ralstonia solanacearum*. With increase in the amount of inhibitor protein in well, the zone of growth inhibition also increased in case of each bacteria **(Plate 8, 9, 10, 11 and 12)**. The zone of growth inhibition in bacterial strain, *Xanthomonas campestris* (8, 10, 11, 16 and 18mm), *Agrobacterium tumefaciens* (6, 8, 12, 13 and 16mm), *Ralstonia solanacearum* (4, 8, 16, 17 and 19mm), *Bacillus subtilis* (7, 13, 14, 15 and 22mm) and *Staphylococcus aureus* (9, 11, 13, 14 and 17mm) was observed when 26, 52, 104, 208 and 416 µg/ml of inhibitor protein was loaded to the well. The results obtained are presented in Table 8.

**Table 8. Effect of partially purified trypsin inhibitor on bacterial growth**

| Inhibitor concentration (µg) | Zone of Bacterial growth inhibition (mm) | | | | |
|---|---|---|---|---|---|
| | ***Xanthomonas campestris*** | ***Agrobacterium tumefaciens*** | ***Ralstonia solanacearum*** | ***Bacillus subtilis*** | ***Staphylococcus aureus*** |
| **26** | 8±0.61 | 6±0.69 | 4±0.85 | 7±0.65 | 9±0.86 |
| **52** | 10±0.55 | 8±0.61 | 8±0.61 | 13±0.76 | 11±0.52 |
| **104** | 11±0.52 | 12±0.79 | 16±0.45 | 14±0.47 | 13±0.73 |
| **208** | 16±0.45 | 13±0.73 | 170.66 | 15±0.69 | 14±0.47 |
| **416** | 18±0.46 | 16±0.45 | 19±0.63 | 22±0.39 | 17±0.40 |
| **$CD_{0.05}$** | 1.65 | 2.14 | 2.09 | 1.96 | 2.01 |

Data represents mean values ± standard error of three values.

It has been proposed that the protein with antibacterial action forms a channel on the cell membrane and the cell dies as a result of the out flowing of cellular contents. Dabhade *et al*. (2016) reported antibacterial activity of nontoxic trypsin inhibitor isolated from *Albizia*

*amara* Boiv. against *Pseudomonas aeruginosa* and *Bacillus subtilis*. Wang *et al*. (2006) isolated protease inhibitor from *Phaseolus mungo* seeds and reported its antibacterial activity towards *Staphylococcus aureus* and *Salmonella typhimurium*. Dokka and Davuluri (2014) reported trypsin inhibitor from seeds of *Abelmoschus moschatus* with antibacterial activity against *Staphyloccocus aureus, Escherichia coli, Proteus vulgaris, Bacillus subtilis, Streptococcus pneumonia, Bacillus aureus.* It was moderately active against *Klebsiella pneumonia.* A novel protease inhibitor named "fistulin" was isolated from leaves of *Cassia fistula*, having significant antibacterial activity against *Staphylococcus aureus, E. coli, Bacillus subtilis* and *Klebsiella pneumonia* (Arulpandi and Sangeetha, 2012). Habib *et al*. (2016) reported antibacterial activity of inhibitor protein isolated from seeds of pea (*Pisum sativum* L.) against *E. coli* (ATCC 25922) strain and reported crude aqueous extract to be highly active in inhibiting the bacterial growth.

### 4.4.2 Anticancerous activity of partially purified trypin inhibitor

Partially purified trypsin inhibitor protein isolated from *Phaseolus vulgaris* L. cultivar Baspa was found to be effective against Hep-2C cell line i.e. Human Cervix Carcinoma cell line (HeLa derivative). To calculate cell proliferation MTT assay was performed. As the concentration of partially purified inhibitor protein was increased in assay the cell viability decreased. The effect of inhibitor protein on Hep-2C cell line growth after 24 hrs of exposure shown in Fig. 3. When the concentration of inhibitor protein was 15µg, the per cent viability of cells were 60.88%. As the concentration of this inhibitor protein was increased upto 120µg the per cent viability of cells were decreased to 25.74%. The results obtained are in Table 9.

**Table 9. Effect of partially purified inhibitor on carcinoma cells**

| Protease Inhibitor protein concentration (µg/ml) | Percent viability of cancerous cells (Hep-2C cell line) |
|---|---|
| **Control** | 100 |
| **15 µg** | 60.88 |
| **30 µg** | 38.57 |
| **45 µg** | 37.59 |
| **60 µg** | 34.60 |
| **75 µg** | 34.44 |
| **90 µg** | 31.73 |
| **105 µg** | 30.57 |
| **120 µg** | 25.74 |

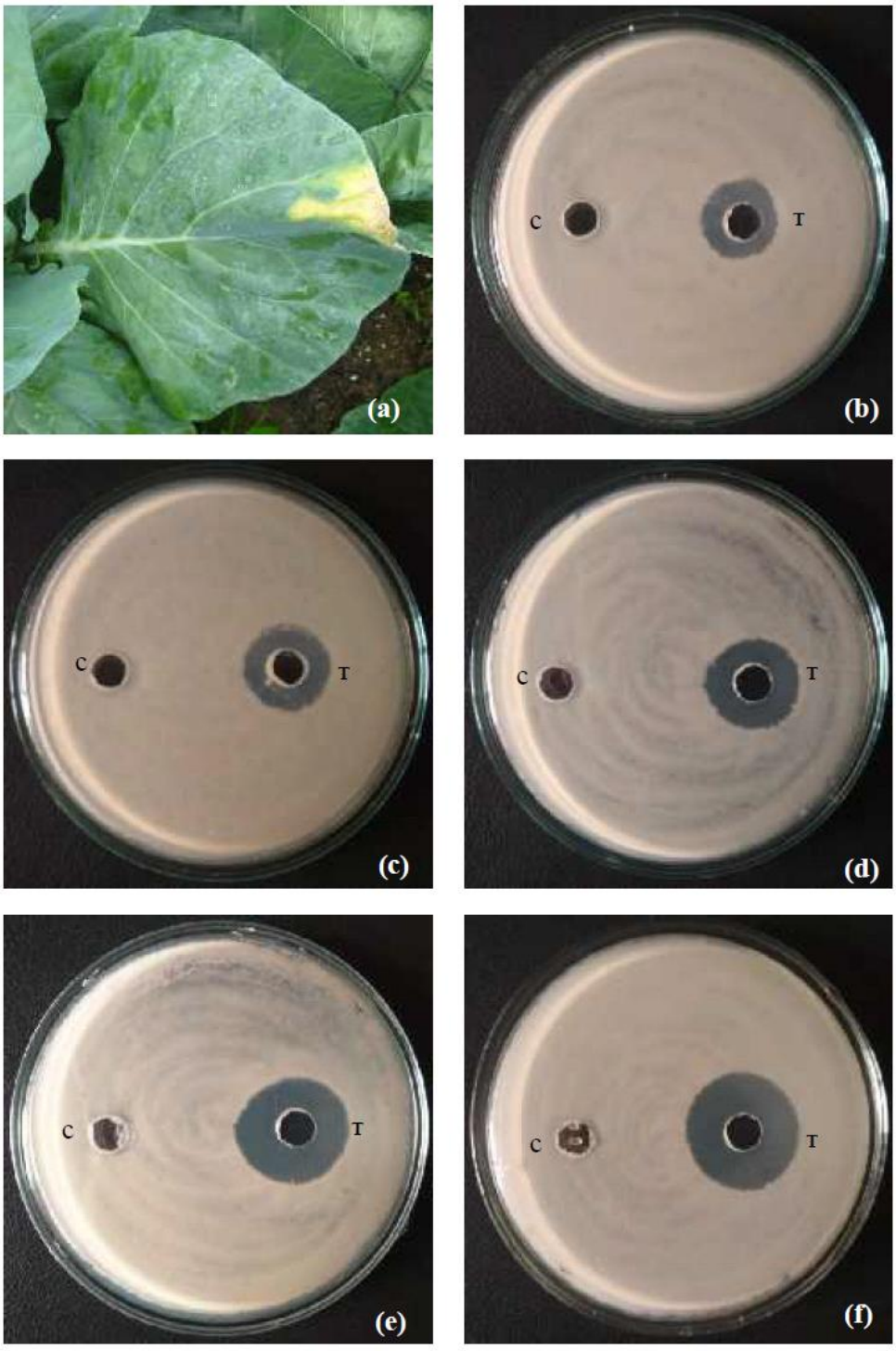


**Plate 8. Antibacterial activity of partially purified inhibitor on *Xanthomonas campestris* growth at different concentrations of inhibitor protein**

(a) V-shaped yellow lesions to margin of leaves of cabbage caused by *Xanthomonas campestris*
Control = 0µg/ml
(b) Inhibitor conc.= 26µg/ml
(c) Inhibitor conc.= 52µg/ml
(d) Inhibitor conc.= 104µg/ml
(e) Inhibitor conc.= 208µg/ml
(f) Inhibitor conc.= 416µg/ml

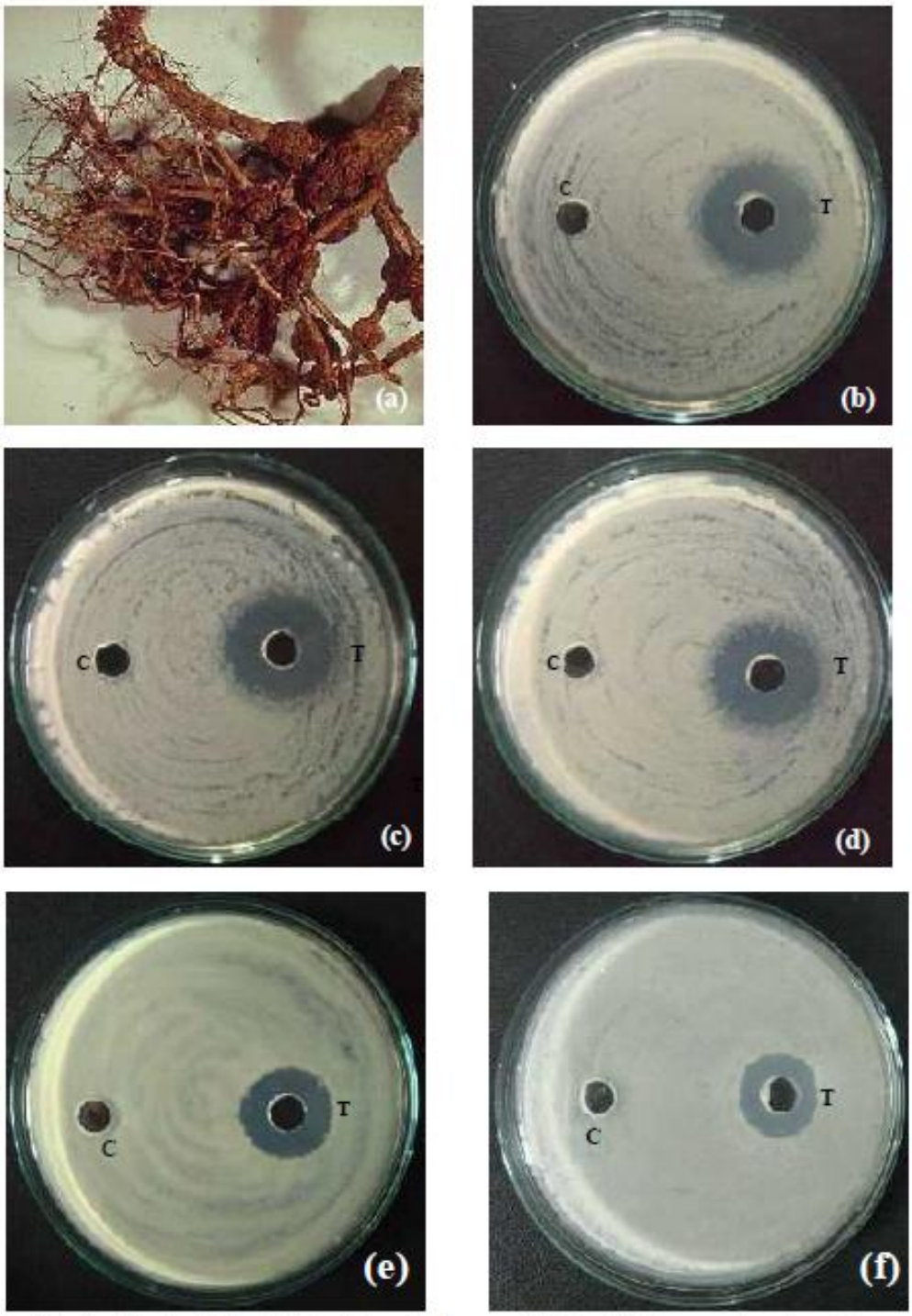


**Plate 9. Antibacterial activity of partially purified inhibitor on *Agrobacterium tumefaciens* growth at different concentrations of inhibitor protein**

(a) Crown gall disease of apple caused by *Agrobacterium tumefaciens* Control = 0µg/ml
(a) Inhibitor conc.= 26µg/ml
(c) Inhibitor conc.= 52µg/ml
(d) Inhibitor conc.= 104µg/ml
(e) Inhibitor conc.= 208µg/ml
(f) Inhibitor conc.= 416µg/ml

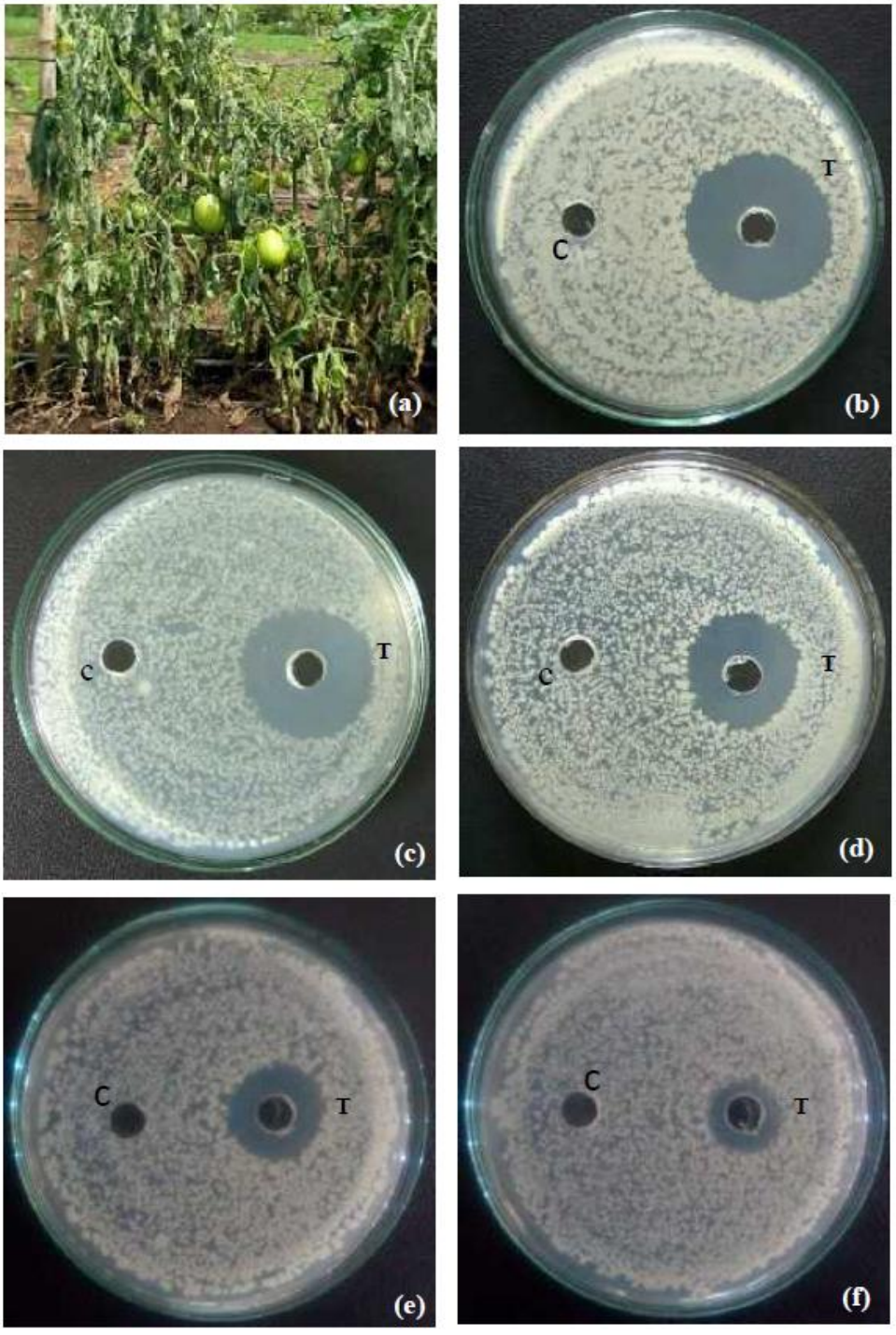


**Plate 10. Antibacterial activity of partially purified inhibitor on *Ralstonia solanacearum* growth at different concentrations of inhibitor protein**

(a) Wilting of tomato leaves without their yellowing caused by *Ralstonia solanacearum*
Control = 0µg/ml
(b) Inhibitor conc.= 26µg/ml
(c) Inhibitor conc.= 52µg/ml
(d) Inhibitor conc.= 104µg/ml
(e) Inhibitor conc.= 208µg/ml
(f) Inhibitor conc.= 416µg/ml

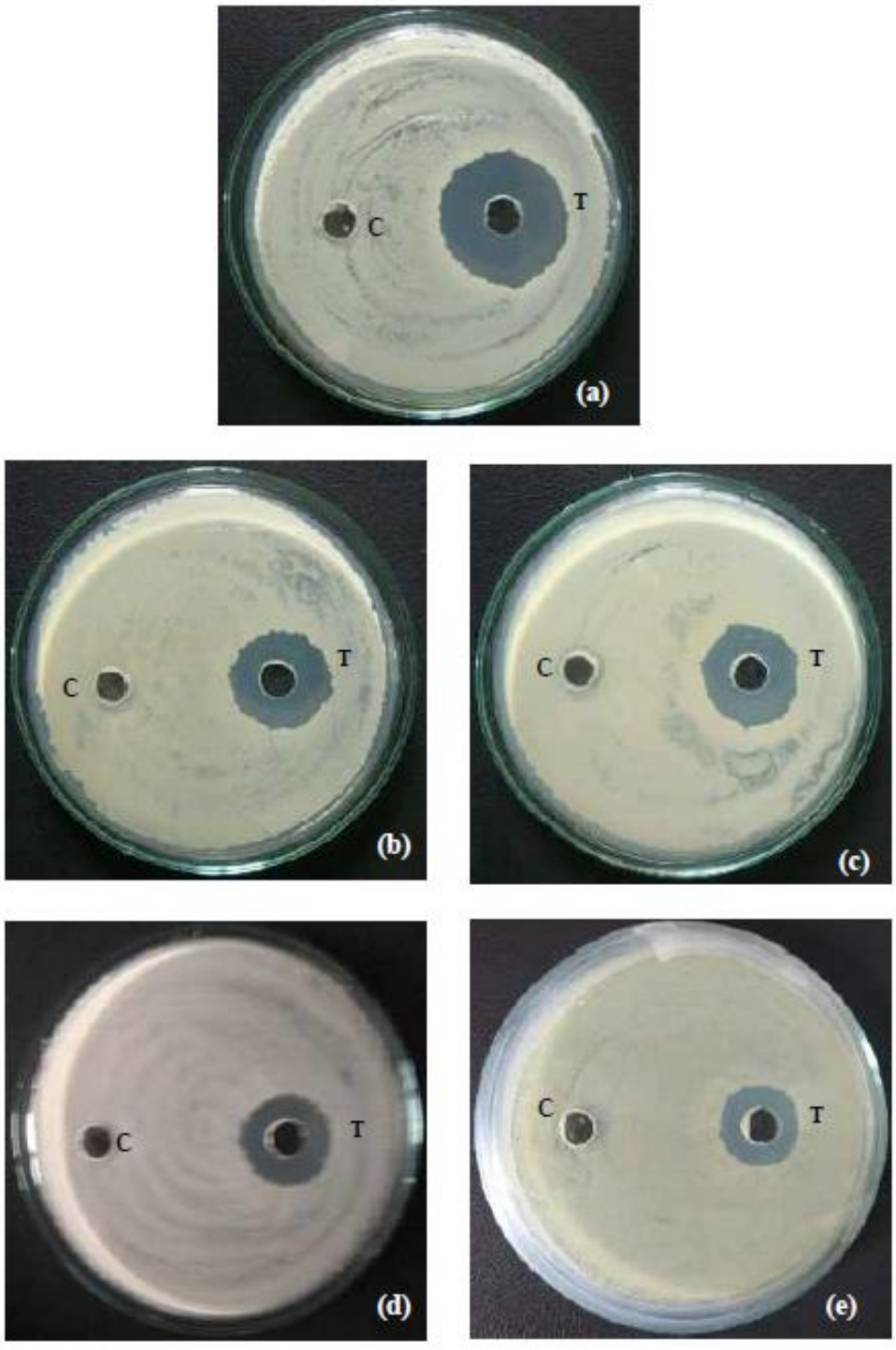


**Plate 11. Antibacterial activity of partially purified inhibitor on *Bacillus subtilis* growth at different concentrations of inhibitor protein**

Control = 0µg/ml
(a) Inhibitor conc.= 26µg/ml
(b) Inhibitor conc.= 52µg/ml
(c) Inhibitor conc.= 104µg/ml
(d) Inhibitor conc.= 208µg/ml
(e) Inhibitor conc.= 416µg/ml

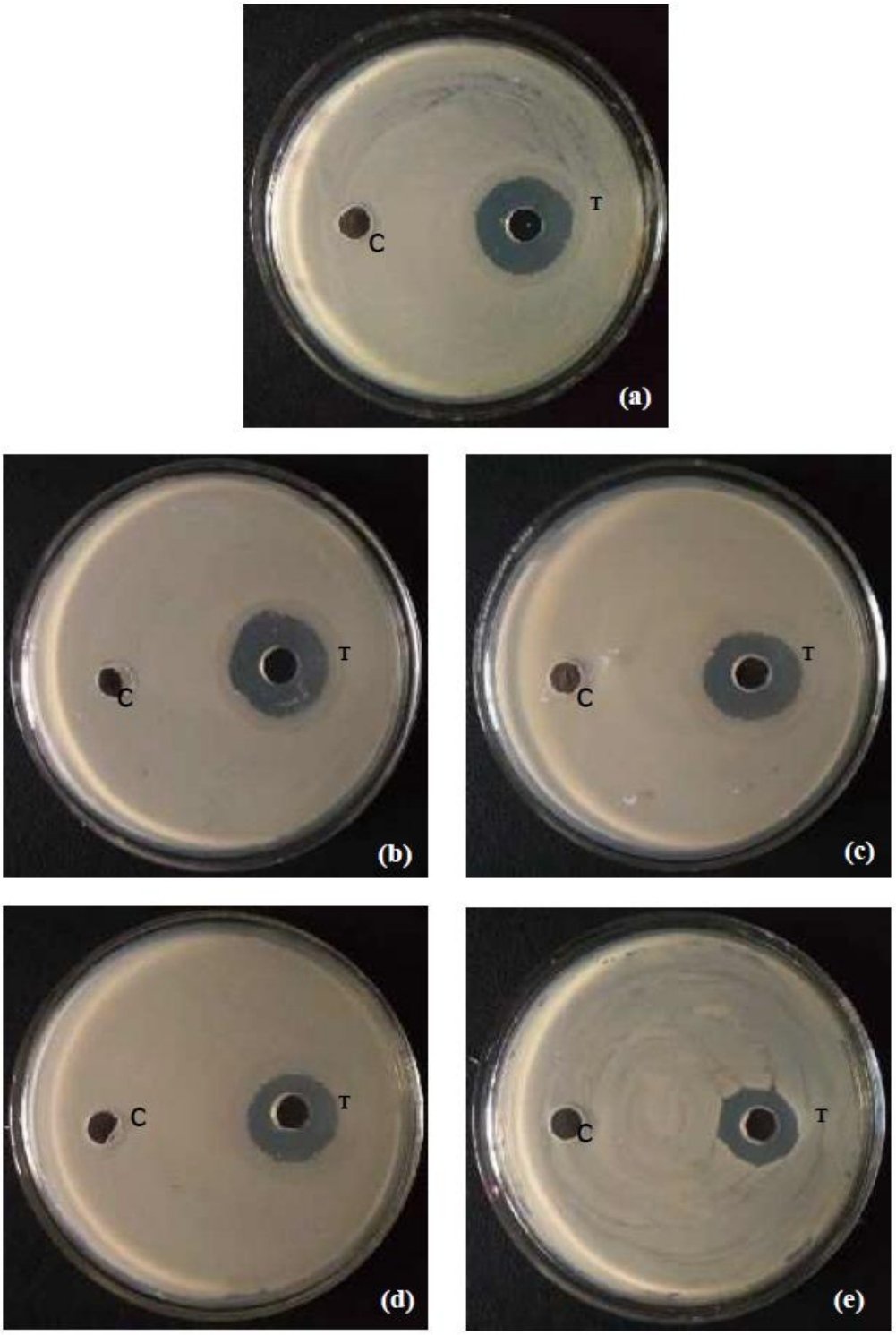


**Plate 12. Antibacterial activity of partially purified inhibitor on *Staphylococcus aureus* growth at different concentrations of inhibitor protein**

Control = 0µg/ml
(a) Inhibitor conc.= 26µg/ml
(b) Inhibitor conc.= 52µg/ml
(c) Inhibitor conc.= 104µg/ml
(d) Inhibitor conc.= 208µg/ml
(e) Inhibitor conc.= 416µg/ml

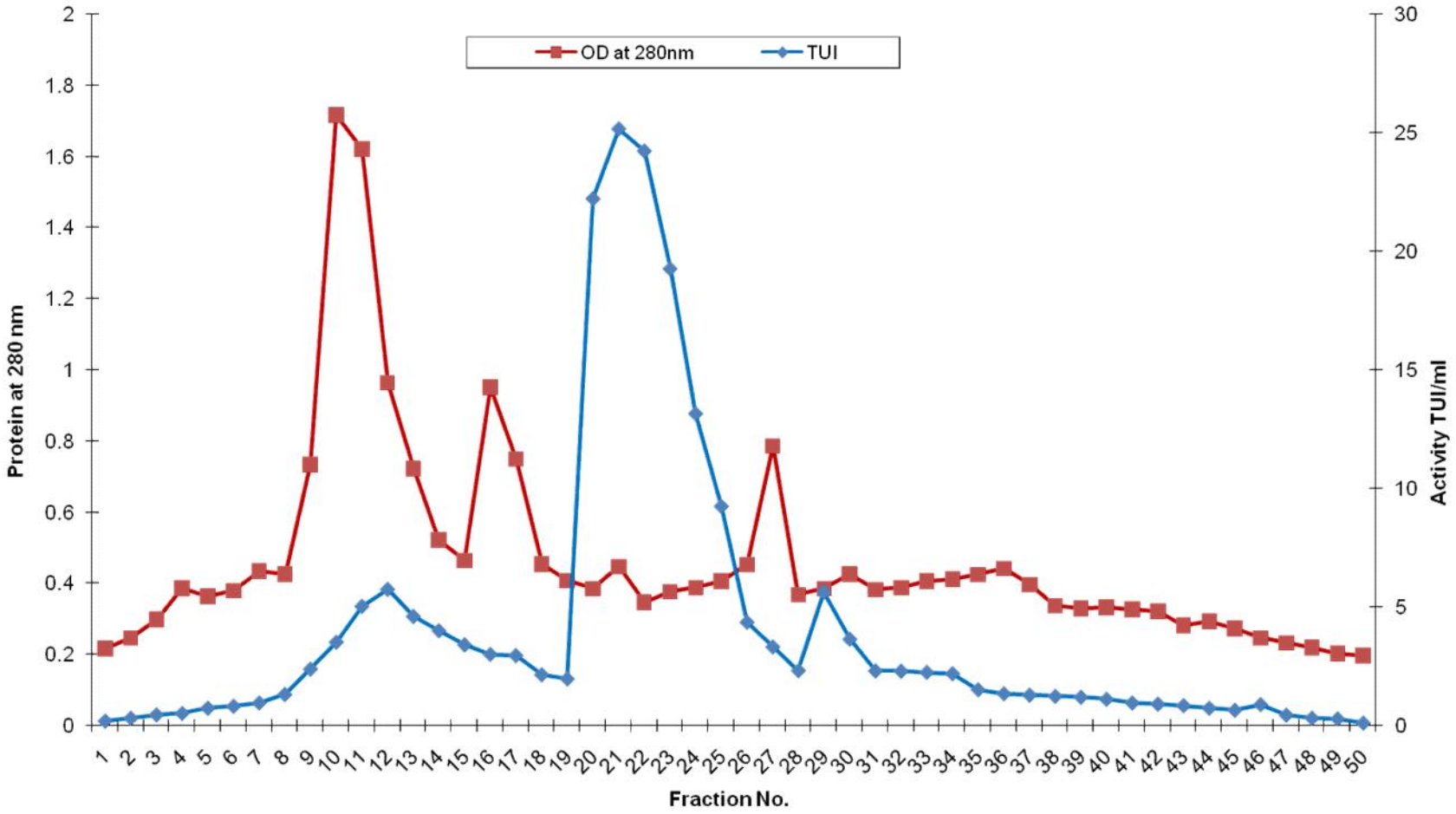


Fig. 2 Elution profile of trypsin inhibitor on Sephadex (G-100) column

Several studies suggested that different legume protein inhibitors (PIs) exhibit potent anticarcinogenic and antiproliferative activity. Wang *et al*. (2006) reported anti-proliferative activity of protease inhibitor isolated from *Phaseolus mungo* against human hepatoma cells Bel-7402. Bezerra *et al*. (2016) reported *Inga vera* trypsin inhibitor (IVTI) from *Inga vera* seeds to reduce the viability of human epithelial colorectal adenocarcinoma cells (CACO-2) to about 70 %. Fang *et al*. (2010) isolated trypsin inhibitor protein from Korean large black soybeans (KBTI) that inhibited HIV-1 reverse transcriptase activity and also reported KBTI to exert weak antiproliferative activity toward CNE-2 and HNE-2 nasopharyngeal cancer cells, MCF-7 breast cancer cells, and Hep G2 hepatoma cells. Bowman-Birk protease inhibitor isolated from *Vigna unguiculata* seeds showed apoptosis and lysosome membrane permeabilization induction on breast cancer cell (Joanitti *et al*., 2010).

# *Chapter-5*

# SUMMARY AND CONCLUSION

The important findings of the present investigation entitled **"Studies on biological activity of Bowman-Birk trypsin inhibitor from a local bean cultivar"** are summarized as follows**:**

**A) Partial purification of trypsin inhibitor protein**

- Maximum trypsin inhibitor protein was extracted in distilled water from the seeds of bean (*Phaseolus vulgaris* L. ) cultivar Baspa.
- Best extraction time for trypsin inhibitor protein was found to be 3 hours.
- The inhibitor was purified to 5.5 folds with 76.5 per cent recovery by ammonium sulfate precipitation (20-80 %) and gel filtration chromatography on Sephadex G-100 column.
- The partially purified inhibitor showed two bands with molecular weight approximately 12000 daltons and 14000 daltons on SDS-PAGE.
- The partially purified inhibitor protein appears to be of Bowman-Birk type.

**B) Biological activity of partially purified inhibitor**

- The partially purified inhibitor protein was found to inhibit the growth of fungal strains viz., *Fusarium oxysporum*, *Colletotricum gloeosporioides, Alternaria solani* and *Cercospora punicae.* Maximum per cent inhibition (88.12 %) was found against fungal strain *Cercospora punicae* in potato dextrose agar (PDA) medium supplemented with 300µg of inhibitor protein in 25ml of medium. In the same medium minimum per cent inhibition was found in strain *Alternaria solani* (79.41 %).
- The partially purified inhibitor protein was also found to inhibit the growth of bacterial strains viz., *Xanthomonas campestris, Agrobacterium tumefaciens, Ralstonia solanacearum, Bacillus subtilis and Staphylococcus aureus.* The inhibitor protein was found highly effective against bacterial strain *Bacillus subtilis* (22mm) in nutrient agar (NA) medium supplemented with 416µg/ml of inhibitor protein and less effective against *Ralstonia solanacearum* (4mm) with 26µg/ml of inhibitor protein .

- ❖ Partially purified inhibitor protein was also found to inhibit the growth of cancerous cells (Hep-2C cell i.e. derivatives of HeLa cells).

## CONCLUSION

Present investigation indicated the purified inhibitor protein to be effective against selected bacterial and fungal plant pathogenic strains with varying efficiencies. So partially purified inhibitor protein can be used in pharmaceuticals as therapeutic agent for emphasis against control of plant pathogens and can also be applied in agricultural sector to control plant diseases caused by pathogenic bacteria and fungus. The partially purified inhibitor protein was also found to be active against proliferation of cancerous cells (Hep-2C cells i.e. derivative of HeLa cells). So it can be used as an effective anticancerous agent in future applications.

# LITERATURE CITED


Alasbahi R and Melzig MF. 2008. Screening of some Yemeni medicinal plants for inhibitory activity against peptidases. *Pharmazie* 63:86-88.

Armstrong WB, Kennedy AR, Wan XS, Atiba J, McLaren E and Meyskens FL. 2000. Single-dose administration of Bowman-Birk inhibitor concentrate in patients with oral leukoplakia. *Cancer Epidemiology, Biomarkers and Prevention* 9:43-47.

Arulpandi I and Sangeetha R. 2012. Antibacterial activity of Fistulin: A protease inhibitor purified from the leaves of *Cassia fistula*. *International Scholarly Research Network Pharmaceutics* 1:1-4.

Barla S, Kaladhar DS and Rao NV. 2016. In vitro antifungal and antiproliferative evaluation of a trypsin inhibitor from testa of *Citrullus lanatus* Linn. *International Journal of Life Sciences and Scientific Research* 2:78-87.

Benjakul S, Visessanguan W and Thummaratwasik P. 2000. Isolation and characterization of trypsin inhibitors from Thai legume seeds. *Journal of Food Biochemistry* 24:107-127.

Bergey DR, Howe GA and Ryan CA. 1996. Polypeptide signaling for plant defensive genes exhibits analogies to defense signaling in animals. *Proceedings of the National Academy of Sciences of the United States of America* 93:12053-12058.

Bezerra CS, Oliveira CFR, Machado OLT, Mello GSV, Pitta MGR, Rego MJBM, Napoleao TH, Paiva PMG, Ribeiro SFF, Gomes VM, Silva ON, Neto SM, Franco OL and Macedo MLR. 2016. Exploiting the biological roles of the trypsin inhibitor from *Inga vera* seeds: A multifunctional Kunitz inhibitor. *Process Biochemistry* 51:792–803.

Bode W and Huber R. 2000. Structural basis of the endoproteinases-protein inhibitor interaction. *Biochimica Biophysica Acta* 1477:241-252.

Chan YS, Zang Y and Ng TB. 2013. Brown kidney bean Bowman-Birk trypsin inhibitor is heat and pH stable and exhibit anti-proliferative activity. *Applied Biochemistry and Biotechnology* 169:1306-1314.

Chan YS, Zhang Y, Sze SCW and Ng TB. 2014. A thermostable trypsin inhibitor with antiproliferative activity from small pinto beans. *Journal of Enzyme Inhibition and Medicinal Chemistry* 29:485-490.

Chandrashekharaiah KS. 2013. Physico-chemical and antifungal properties of trypsin inhibitor from the seeds of *Mucuna pruriens. Oriental Journal of Chemistry* 29:1061-1070.

Chaudhary NS, Shee C, Islam A, Ahmad F, Yernool D, Kumar P and Sharma AK. 2008. Purification and characterization of a trypsin inhibitor from *Putranjiva roxburghii* seeds. *Phytochemistry* 69:2120–2126.

Chilosi G, Caruso C, Caporale C, Leonardi L, Bertini L, Buzi A, Nobile M, Magro P and Buonocore V. 2000. Antifungal activity of a Bowman-Birk ype trypsin inhibitor from wheat kernel. *Journal of Phytopathology* 148:477-481.

Coscueta ER, Pintado ME, Pico GA, Knobel G, Boschetti CE, Malpiedi LP and Nerli BB. 2017. Analytical methods continuous method to determine the trypsin inhibitor activity in soybean flour. *Food Chemistry* 214:156-163.

Dabhade AR, Mokashe NU and Patil UK. 2016. Purification, characterization and antimicrobial activity of nontoxic trypsin inhibitor from *Albizia amara* Boiv. *Process Biochemistry* 51:659-674.

Deshimaru M, Hanamoto R, Kusano C, Yoshimi S and Terada S. 2002. Purification and characterization of proteinase inhibitors from wild soja (*Glycine soja*) seeds. *Bioscience, Biotechnology and Biochemistry* 66:1897-1903.

Dias GB, Gomes VM, Pereira UZ, Ribeiro SFF, Carvalho AO, Rodrigues R, Machado OLT, Fernandes KVS and Ferreira ATS. 2012. Isolation, characterization and antifungal activity of proteinase inhibitors from *Capsicum chinense* Jacq. seeds. *The Protein Journal* 32:15-26.

Dokka MR and Davuluri SP. 2014. Antimicrobial activity of trypsin inhibitor from the seeds of *Abelmoschus moschatus* L. *International Journal of Current Microbiology and Applied Sciences* 3:184-199.

Fang EF, Hassanien AE, Wong JH, Bah CSF, Soliman SS and Ng TB. 2010. Purification and modes of antifungal action by *Vicia faba* cv. egypt trypsin inhibitor. *Journal of Agriculture Food Chemistry* 58:10729-10735.

Ferlay J, Soerjomataram I, Dikshit R, Eser S, Mather C, Rebelo M, Parkin DM, Forman D and Bray F. 2015. Cancer incidence and mortality worldwide: sources, methods and major pattern in GLOBOCAN 2012. *International Journal of Cancer* 136:359-386.

Gauthier GM and Keller NP. 2013. Cross over fungal pathogens: The biology and pathogenesis of fungi capable of crossing kingdoms to infect plants and humans. *Fungal Genetics and Biology* 61:146-157.

Godbole SA, Krishna TG and Bhatia CR. 1994. Purification and characterization of protease inhibitor from pigeon pea (*Cajanus cajan* (L) millsp) seeds. *Journal of the Science of Food and Agriculture* 64:87-93.

Gomez KA and Gomez AA. 1984. Statistical procedures for agricultural research. John Willey and Sons, New York. 690p.

Gu C, Song X, Zhao L, Pan S and Qin G. 2014. Purification and characterization of Bowman-Birk trypsin inhibitor from soybean. *Journal of Food and Nutrition Research* 2:546-550.

Guillamon E, Pedrosa MM, Burbano C, Cuadrado C, Sanchez MC and Muzquiz M. 2008. The trypsin inhibitors present in seed of different grain legume species and cultivar. *Food Chemistry* 107:68-74.

Gupta P, Dhawan K, Malhotra SP and Singh R. 2000. Purification and characterization of trypsin inhibitor from seeds of faba bean (*Vicia faba* L.). *Acta Physiologia Plantarum* 22:433-438.

Habib H, Fazili KM, Zargar MA and Ganie BA. 2016. Protease inhibitor associated antimicrobial activity of pea *Pisum sativum* L. cv. Arkel. *International Journal of Pure and Applied Biosciences* 4:172-179.

Hermosa MR, Turra D, Fogliano V, Monte E and Lorito M. 2006. Identification and characterization of potato protease inhibitors able to inhibit pathogenicity and growth of *Botrytis cinerea*. *Physiological and Molecular Plant Pathology* 68:138-148.

Hong Y, Cai X, Shao B, Hong J, Wang S and Rao P. 2013. Isolation of a thermostable trypsin inhibitor with exploitable potential. *European Food Research and Technol*ogy 237:457-465.

Joanitti GA, Azevedo RB and Freitas SM. 2010. Apoptosis and lysosome membrane permeabilization induction on breast cancer cells by an anticarcinogenic Bowman-Birk protease inhibitor from *Vigna unguiculata* seeds. *Cancer Letters* 293:73-81.

Joshi B, Sainani M, Bastawade K, Gupta VS and Ranjekar PK. 1998. Cysteine protease inhibitor from pearl millet: A new class of antifungal protein. *Biochemical Biophysics Research Communication* 246:382-387.

Kalashnikova EE, Chernyshova MP and Iqnatov VV. 2003. The extracellular proteases of the phytopathogenic bacterium *Xanthomonas campestris*. *Mikrobiologiia* 72:498-502.

Kennedy AR. 1993. Cancer prevention by protease inhibitors. *Preventing Medication* 22:796-811.

Klomklao S, Benjakul S, Kishimura H and Chaijan M. 2011. Extraction, purification and properties of trypsin inhibitor from Thai mung bean (*Vigna radiate* L.). *Food Chemistry* 129:1348-1354.

Kuhar K, Mittal A, Kansal R and Gupta VK. 2014. Purification of a protease inhibitor from *Dolichos biflorus* using immobilized metal affinity chromatography. *Indian Journal of Biochemistry and Biophysics* 51:66-74.

Kumar P, Sreerama YN and Gowda LR. 2002. Formation of Bowman-Birk inhibitors during germination of *Dolichos biflorus*. *Phytochemistry* 60:581-588.

Laemmli UK. 1970. Cleavage of structure proteins during the assembly of the head of bacteriophage T4. *Nature* 227: 680-685**.**

Lanza E, Hartman TJ, Albert PS, Shields R, Slattery M and Caan B. 2006. High dry bean intake and reduced risk of advanced colorectal adenoma recurrence among participants in the polyp prevention trial. *Journal of Nutrition* 136:1896-1903.

Laskowski M and Qasim MA. 2000. What can the structure of enzyme-inhibitor complexes tells us about the structure of the enzyme substrate complexes? *Biochimica Biophysica Acta* 1477:324-337.

Li M , Liu Q, Cui Y, Li D, Wang H and Ng TB. 2017. Isolation and characterization of a *Phaseolus vulgaris* trypsin inhibitor with antiproliferative activity on leukemia and lymphoma cells. *Molecules* 22:187.

Lima AIG, Mota J, Monteiro SAVS and Ferreira RMSB. 2016. Legume seeds and colorectal cancer revisited: protease inhibitor reduce MMP-9 activity and colon cancer cell migration. *Food Chemistry* 197:30-38.

Lopes JLS, Valadares NF, Moraes DI, Rosa JC, Araujo HSS and Beltramini LM. 2009. Physico-chemical and antifungal properties of protease inhibitors from *Acacia plumose. Phytochemistry* 70:871-879.

Lowry OH, Roseburgh NJ, Farr AK and Randall RJ. 1951. Protein measurement with Folin's phenol reagent. *Journal of Biological Chemistry* 193:265-275.

Macedo MLR, Filho EBSD, Freire MGM, Oliva MLV, Sumikawa JT, Toyama MH and Marangoni S. 2011. Trypsin inhibitor from *Sapindus saponaria* L. seeds: purification, characterization and its activity towards insect pest digestive enzyme. *Journal of Protein* 30:9-19.

Macedo MLR, Ribeiro SFF, Taveira GB, Gomes VM, de Barros KMCA and Neto SM. 2016. Antimicrobial activity of ILTI, a Kunitz-Type trypsin inhibitor from *Inga laurina* (SW.) Willd. *Current Microbiology* 72:538-544.

Mirjana BP, Radovic BV, Barac MB, Stanojevic SP and Nedovic VA. 2007. Influence of different genotypes on trypsin inhibitor levels and activity in soybean. *Sensors* 7:67-74.

Mojica L and Mejia EG. 2015. Characterization and comparison of protein and peptide profiles and their biological activities of improved common bean. *Plant Foods for Human Nutrition* 70:105-112.

Mourao CBF and Schwartz EF. 2013. Protease inhibitors from marine venomous animals and their counterparts in terrestrial venomous animals. *Marine Drugs* 11:2069-2112.

Nair M and Sandhu SS. 2013. A Kunitz trypsin inhibitor from chickpea (*Cicer arietinum* L.) that exerts an antimicrobial effect on *Fusarium oxysporum* f.sp. *ciceris. Agricultural Sciences* 4:585-594.

Nath AK, Kumari R and Sharma S. 2014. Trypsin inhibitor activity in developing and germinating seeds of *Dolichos biflorus* L. *Journal of Food Legumes* 27:264-267.

Ng TB, Lam SK and Fong WP. 2003. A homodimeric sporamin-type trypsin inhibitor with antiproliferative, HIV reverse transcriptase-inhibitory and antifungal activities from Wampee (*Clausena lansium*) seeds. *Biological Chemistry* 384:289-293.

Oerke EC and Dehne HW. 2004. Safeguarding production-losses in major crops and the role of crop protection. *Crop Protection* 23:275-285.

Oerke EC, Dehne HW, Schonbeck F and Weber A. 1994. *Crop production and crop protection: estimated losses in major food and cash crops*, Elsevier, Amsterdam.

Pesoti AR, Oliveira BM, Oliveira AC, Pompeu DG, Goncalves DB, Marangoni S, Silva JA and Granjeiro PA. 2015. Extraction, purification and characterization of inhibitor of trypsin from *Chenopodium quinoa* seeds. *Food Science and Technology* 35:588-597.

Prasad ER, Gupta AD and Padmasree K. 2010. Purification and characterization of a Bowman-Birk proteinase inhibitor from the seeds of black gram (*Vigna mungo*). *Phytochemistry* 71:363-372.

Raj Deepika and Nath AK. 2010. Studies on trypsin inhibitor in bean (*Phaseolus vulgaris* L.) cultivars of Himalayan region. *Journal of Plant Biochemistry and Biotechnology* 19:103-105.

Raj Deepika, Nath A and Nath AK. 2008. Toxicity of trypsin inhibitor from *Phaseolus vulgaris* L. cultivar against *Pieris brassicae*. *Pest Technology* 2:32-37.

Rakashanda S, Ishaq M, Masood A and Amin S. 2012. Antibacterial activity of a Trypsin-Chymotrypsin-Elastase inhibitor isolated from *Lavatera cashmeriana* Camb seeds. *The Journal of Animal and Plant Sciences* 22:983-986.

Sammour RHA. 2005. Isolation and characterization of four isoinhibitors from cowpea (*Vigna unguiculata* (L.) walp) seeds. *Journal of Biology* 30:207-215.

Satheesh LS and Murugan K. 2011. Antimicrobial activity of protease inhibitor from leaves of *Coccinia grandis* (L.) Voigt. *Indian Journal of Experimental Biology* 49:366-374.

Satheesh S and Murugan K. 2012. Protease inhibitor from Coccinia grandis (L.) Voigt. leaves: purification, characterization and kinetic properties. *International Journal of Pharmacy and Pharmaceutical Sciences* 4:565-573.

Scarafoni A, Consonni A, Galbusera V, Negri A, Tedeschi G, Rasmussen P, Magni C and Duranti M. 2008. Identification and characterization of a Bowman-Birk inhibitor active towards trypsin but not chymotrypsin in *Lupinus albus* seeds. *Journal of Phytochemistry* 69:1820-1825.

Sessa DJ and Wolf WJ. 2001. Bowman-Birk inhibitors in soybean seed coats. *Industrial Crops and Products* 14:73-83.

Shah KR, Patel DK, Pappachan A, Prabha CR and Singh DD. 2016. Characterization of Kunitz-type serine protease inhibitor from *Solanum tuberosum* having lectin activity. *International Journal of Biological Macromolecules* 83:259-269.

Shamsi TN, Parveen R and Fatima S. 2017. Trypsin inhibitors demonstrate antioxidant activities, inhibit A549 cell proliferation, and increase activities of reactive oxygen species scavenging enzymes. *Indian Journal of Pharmacology* 49:155-160.

Sharma P, Nath AK, Kumari R and Bhardwaj SV. 2012. Purification, characterization and evaluation of insecticidal activity of trypsin inhibitor from *Albizia lebbeck* seeds. *Journal of Forestry Research* 23:131-137.

Sharma S. 2013. Studies on trypsin inhibitor in local cultivar of *Dolichos biflorus* L. M.Sc. Thesis, Dr. Y.S. Parmar University of Horticulture and Forestry, Nauni, Solan (HP)

Sharma U and Suresh CG. 2011. Purification, crystallization and X-ray characterization of a Kunitz-type trypsin inhibitor protein from the seeds of chickpea (*Cicer arietinum*). *Acta Crystallographica Section F: Structural Biology and Crystallization Communications* 67:714-717.

Shulke RH and Murdock LS. 1983. Lipoxygenase trypsin inhibitor and lectin from soybean: effects on larval growth of *Menduca sexta* (Lepidoptera, Sphingidaer). *Environmental Entomology* 12:787-791.

Sun J, Wang H and Ng TB. 2010. Trypsin isoinhibitors with antiproliferative activity toward leukemia cells from *Phaseolus vulgaris* cv "White Cloud Bean". *Journal of Biomedicine and Biotechnology* 1:1-8.

Swathi M, Lokya V, Swaroop V, Mallikarjuna N, Kannan M, Dutta AG and Padmasree K. 2014. Structural and functional characterization of proteinase inhibitor from seeds of *Cajanus cajan* (cv. ICP 7118). *Plant Physiology and Biochemistry* 83:77-87.

Terras FRG, Torrekens S, Leuvenb FV, Osborn RW, Vanderleyden J, Cammuuea BPA and Broekaerta WF. 1993. A new family of basic cysteine rich plant antifungal protein from Brassicaceae species. *Federation of European Biochemical Societies* 3:233-240.

Thakur A. 2004. *Studies on trypsin inhibitor in pea cultivars.* M.Sc. Thesis, Dr. Y. S. Parmar University of Horticulture and Forestry, Nauni, Solan (HP)

Thakur N, Chand S and Nath AK. 2015. Protease inhibitor activity in seed flour of local bean (*Phaseolus vulgaris* L.) cultivars. *Journal of Food Legumes* 28:262-264.

Tian Y and Zhang K. 2005. Purification and characteristic of proteinase inhibitor GLPIA2 from *Ganoderma lucidum* by submerged fermentation. *Chinese Journal of Chromatography* 23:267-269.

Vernekar JV, Tanksale AM, Ghatge MS and Deshpande VV. 2001. Novel bifunctional alkaline protease inhibitor: protease inhibitory activity as the biochemical basis of antifungal activity. *Biochemical and Biophysical Research Communications* 285:1018-1024.

Wang HX and Ng TB. 2007. Isolation and characterization of an antifungal peptide with antiproliferative activity from seeds of *Phaseolus vulgaris* cv. 'Spotted Bean'. *Applied Microbiology and Biotechnology* 74:125-130.

Wang S and Rao P. 2010. A leguminous trypsin-chymotrypsin inhibitor Limenin with antifungal activity from *Phaseolus limensis*. *European Food Research and Technology* 231:331-338.

Wang S, Lin J, Ye M, Ng TB, Rao P and Ye X. 2006. Isolation and characterization of a novel bean protease inhibitor with antipathogenic and antiproliferative activities. *Peptides* 27:3129-3136.

Wasko A, Kieliszek M and Targonski Z. 2012. Purification and characterization of a proteinase from the probiotic *Lactobacillus rhamnosus* OXY. *Preparative Biochemistry and Biotechnology* 42:476-488.

Wati RK, Theppakorn T, Benjakul S and Rawdkuens S. 2010. Trypsin Inhibitor from 3 legume seeds: fractionation and proteolytic inhibition study. *Journal of Food Science* 75:223-228.

Wong JH, Xia L and Ng TB. 2007. A review of defensins of diverse origins. *Current Protein and Peptide Science* 8:446-459.

Ye XY, Ng TB and Rao PF. 2001. Bowman-Birk type trypsin/chymotrypsin inhibitor from broad beans. *Biochemical and Biophysical Research Communications* 289:91-96.

Yili A, Waili A, Aisa HA, Maksimov V, Ziyavitdinov J, Mamadrahimov A, Veshkurova O and Salikhov S. 2015. Purification and characterization of trypsin inhibitor from the *Cicer arietinum* L. (Chickpea) sprouts. *International Journal of Plant Biology & Research* 3:1040.

Ying L. 2009. Isolation and purification of trypsin inhibitor from *Phaseolus vulgaris*. *Journal of Anhui Agricultural Sciences* 9:15-25.

Zhang B, Wong DF, Fan Y, Zhnag Li and Luo Y. 2009. Affinity purification of trypsin inhibitor with anti-*Aspergillus flavus* activity from cultivated and wild soybean. *Mycopathologia* 167:163-171.

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## ABSTRACT

The aim of the present study was to determine the biological activity associated with trypsin inhibitor isolated and purified from the seeds of local bean (*Phaseolus vulgaris* L.) cultivar Baspa. Trypsin inhibitor from local bean seeds showed highest activity in distilled water pH 7.0 in 3 hour of extraction time. Inhibitor protein was partially purified to 5.5 fold with 76.5 per cent recovery by ammonium sulfate precipitation and gel filtration on Sephadex G-100 column. The partially purified inhibitor protein showed two bands with molecular weight approximately 12000 Daltons and 14000 Daltons on SDS-PAGE. The partially purified inhibitor protein was found to inhibit the growth of fungal strains viz., *Fusarium oxysporum*, *Colletotrichum gloeosporioides*, *Alternaria solani* and *Cercospora punicae.* Maximum inhibition of fungal growth in presence of inhibitor protein (300µg) was found in *Cercospora punicae* (88.12%) and minimum growth inhibition was found for *Alternaria solani* (79.41%) in same inhibitor concentration. The partially purified inhibitor protein could also inhibit the growth of bacterial strains viz., *Xanthomonas campestris, Agrobacterium tumefaciens, Ralstonia solanacearum, Bacillus subtilis and Staphylococcus aureus.* The inhibitor protein was found to be highly effective against bacterial strain *Bacillus subtilis* (22mm) and less effective against *Ralstonia solanacearum* (4mm). The effect of inhibitor protein was seen on the growth of cancerous cells (Hep-2C cell i.e. Human Cervix Carcinoma cell line). The results indicate that it inhibited the proliferation of carcinoma cells. Hence it can be applied in agricultural sector to develop transgenics against plant diseases caused by pathogenic microbes and can also be used as an effective anticancerous agent in future applications.

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## APPENDIX-1

**Composition of Nutrient Agar (NA w/l% peptone) medium**

| Constituents | Amount (g/l) |
|---|---|
| **Peptic digest of animal tissue** | 10.0 |
| **Beef extract** | 5.0 |
| **Sodium chloride** | 5.0 |
| **Agar** | 15.0 |

## APPENDIX-II

**Composition of Nutrient Broth (NB) medium**

| Constituents | Amount (g/l) |
|---|---|
| **Peptone** | 10.0 |
| **Beef extract** | 10.0 |
| **Sodium chloride** | 5.0 |

## APPENDIX-III

**Composition of Potato Dextrose Agar (PDA) medium**

| Constituents | Amount (g/l) |
|---|---|
| **Infusion from potato** | 200.0 |
| **Dextrose** | 20.0 |
| **Agar** | 15.0 |

## APPENDIX-IV

**Table 2 ANNOVA table for effect of different extraction media on trypsin inhibitor activity:**

**Total inhibitor activity (TUI/g seed weight)**

| Source | DF | Sum of Square | Mean sum of Squares | F-value |
|---|---|---|---|---|
| Treatment | 3 | 3,241,337.366 | 1,080,445.789 | 165,814.327 |
| Error | 8 | 52.128 | 6.516 | |
| Total | 11 | 3,241,389.494 | | |

**Total soluble protein (mg/g seed weight)**

| Source | DF | Sum of Square | Mean sum of Square | F-value |
|---|---|---|---|---|
| Treatment | 3 | 763.251 | 254.417 | 887.701 |
| Error | 8 | 2.293 | 0.287 | |
| Total | 11 | 765.544 | | |

**Specific inhibitor activity (TUI/mg protein)**

| Source | DF | Sum of Square | Mean sum of Square | F-value |
|---|---|---|---|---|
| Treatment | 3 | 62.378 | 20.793 | 491.513 |
| Error | 8 | 0.338 | 0.042 | |
| Total | 11 | 62.716 | | |

**Table 3 ANNOVA table for effect of extraction time on trypsin inhibitor activity**

**Total inhibitor activity (TUI/g seed weight)**

| Source | DF | Sum of Square | Mean sum of Squares | F-value |
|---|---|---|---|---|
| Treatment | 3 | 4,070,005.180 | 1,356,668.393 | 5,811,875.873 |
| Error | 8 | 1.867 | 0.233 | |
| Total | 11 | 4,070,007.048 | | |

**Total soluble protein (mg/g seed weight)**

| Source | DF | Sum of Square | Mean Square | F-value |
|---|---|---|---|---|
| Treatment | 3 | 2,254.892 | 751.631 | 18,584.517 |
| Error | 8 | 0.324 | 0.040 | |
| Total | 11 | 2,255.216 | | |

**Specific inhibitor activity (TUI/mg protein)**

| Source | DF | Sum of Square | Mean Sum of Square | F-value |
|---|---|---|---|---|
| Treatment | 3 | 4,070,005.180 | 1,356,668.393 | 5,811,875.873 |
| Error | 8 | 1.867 | 0.233 | |
| Total | 11 | 4,070,007.048 | | |

**Table 6 ANNOVA table for effect of partially purified trypsin inhibitor on fungal growth**

**Per cent inhibition of fungal growth (%) of *Fusarium oxysporum***

| Source | DF | Sum of Square | Mean Sum of Square | F-value |
|---|---|---|---|---|
| Treatment | 4 | 3,453.062 | 863.265 | 9,604.811 |
| Error | 10 | 0.899 | 0.090 | |
| Total | 14 | 3,453.960 | | |

**Per cent inhibition of fungal growth (%) of *Colletotricum gloeosporioides***

| Source | DF | Sum of Square | Mean Sum of Square | F-value |
|---|---|---|---|---|
| Treatment | 4 | 1,895.478 | 473.869 | 3,279.372 |
| Error | 10 | 1.445 | 0.145 | |
| Total | 14 | 1,896.923 | | |

**Per cent inhibition of fungal growth (%) of *Alternaria solani***

| Source | DF | Sum of Square | Mean sum of Square | F-value |
|---|---|---|---|---|
| Treatment | 4 | 3,713.544 | 928.386 | 11,676.448 |
| Error | 10 | 0.795 | 0.080 | |
| Total | 14 | 3,714.339 | | |

**Per cent inhibition of fungal growth (%) of *Cercospora punicae***

| Source | DF | Sum of Square | Mean Sum of Square | F-value |
|---|---|---|---|---|
| Treatment | 4 | 3,656.794 | 914.198 | 7,336.662 |
| Error | 10 | 1.246 | 0.125 | |
| Total | 14 | 3,658.040 | | |

**Table 8. ANNOVA table for effect of partially purified trypsin inhibitor on bacterial growth**

**Zone of Bacterial growth inhibition (mm) *Xanthomonas campestris***

| Source | DF | Sum of Square | Mean Sum of Square | F-value |
|---|---|---|---|---|
| Treatment | 4 | 158.619 | 39.655 | 49.100 |
| Error | 10 | 8.076 | 0.808 | |
| Total | 14 | 166.695 | | |

**Zone of Bacterial growth inhibition (mm) of *Agrobacterium tumefaciens***

| Source | DF | Sum of Square | Mean Sum of Square | F-value |
|---|---|---|---|---|
| Treatment | 4 | 173.113 | 43.278 | 32.163 |
| Error | 10 | 13.456 | 1.346 | |
| Total | 14 | 186.569 | | |

**Zone of Bacterial growth inhibition (mm) of *Ralstonia solanacearum***

| Source | DF | Sum of Square | Mean sum of Square | F-value |
|---|---|---|---|---|
| Treatment | 4 | 465.637 | 116.409 | 90.358 |
| Error | 10 | 12.883 | 1.288 | |
| Total | 14 | 478.520 | | |

**Zone of Bacterial growth inhibition (mm) of *Bacillus subtilis***

| Source | DF | Sum of Square | Mean sum of Square | F-value |
|---|---|---|---|---|
| Treatment | 4 | 247.549 | 61.887 | 54.777 |
| Error | 10 | 11.298 | 1.130 | |
| Total | 14 | 258.847 | | |

**Zone of Bacterial growth inhibition (mm) of *Staphylococcus aureus***

| Source | DF | Sum of Square | Mean sum of Square | F-value |
|---|---|---|---|---|
| Treatment | 4 | 76.463 | 19.116 | 16.029 |
| Error | 10 | 11.926 | 1.193 | |
| Total | 14 | 88.388 | | |

# BRIEF BIO-DATA

| | | |
|---|---|---|
| **Name** | **:** | Yamini Thakur |
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**(Yamini Thakur)**